Identity, proliferation capacity, genomic stability and novel senescence markers of mesenchymal stem cells isolated from low volume of human bone marrow

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ABSTRACT

Human bone marrow mesenchymal stem cells (hBM-MSCs) hold promise for treating incurable diseases and repairing of damaged tissues. However, hBM-MSCs face the disadvantages of painful invasive isolation and limited cell numbers. In this study we assessed characteristics of MSCs isolated from residual human bone marrow transplantation material and expanded to clinically relevant numbers at passages 3-4 and 6-7. Results indicated that early passage hBM-MSCs are genomically stable and retain identity and high proliferation capacity. Despite the chromosomal stability, the cells became senescent at late passages, paralleling the slower proliferation, altered morphology and immunophenotype. By qRT-PCR array profiling, we revealed 13 genes and 33 miRNAs significantly differentially expressed in late passage cells, among which 8 genes and 30 miRNAs emerged as potential novel biomarkers of hBM-MSC aging. Functional analysis of genes with altered expression showed strong association with biological processes causing cellular senescence. Altogether, this study revives hBM as convenient source for cellular therapy. Potential novel markers provide new details for better understanding the hBM-MSC senescence mechanisms, contributing to basic science, facilitating the development of cellular therapy quality control, and providing new clues for human disease processes since senescence phenotype of the hematological patient hBM-MSCs only very recently has been revealed.

INTRODUCTION

Human mesenchymal stem cells (hMSCs) are non-hematopoietic, adherent fibroblast-like cells with intrinsic ability of self-renewal and potential for multilineage differentiation [1]. The stromal compartment of bone marrow (BM) was the first biological material from which MSCs were isolated. Since then, BM-derived MSCs have been the most widely studied and are thought to be key regulators of BM physiology [2].

MSCs are the major stem cells for cell therapy and have been used in the clinic for approximately 10 years [3]. Currently, BM represents the major source of MSCs for clinical use [4]. Stem cell-based therapy using human BM-MSCs (hBM-MSCs) holds promise for treating degenerative diseases, cancer, and repair of damaged tissues, where limited therapeutic options exist [5]. E.g., Wernicke et al. reported a high (73.8%) overall response to MSC therapy of the life-threatening severe steroid-refractory graft *versus* host disease [6]. Disadvantage of using hBM-MSCs is the limited cell numbers obtained from invasive isolation techniques [7]. This has led many researchers to investigate alternate sources of human MSCs, including adipose tissue [8] and umbilical cord [9], that can be used in the clinical setting.

High quantities of MSCs are needed for clinical applications, thus requiring extensive cell expansion in long-term culture [10]. However, the occurrence of karyotypic instability in cultured hBM-MSCs has been documented. It has been admitted that genome instability enables tumor cells to acquire their characteristics [11],

therefore the tumorigenesis potential of the hMSCs has become the most important concern for clinical use of MSCs [12]. Though, hBM-MSC studies presented highly conflicting results. It has been shown that hBM-MSCs *in vitro* acquire chromosomal aberrations, undergo spontaneous transformation and form tumors *in vivo* [13]. In contrast, other groups have documented normal karyotype throughout hBM-MSC culture and no malignant transformation *in vivo* [14, 15]. Besides, it has been shown that hBM-MSCs do not transform spontaneously *in vitro* and chromosomal instability occurs without leading to malignant transformation, possibly being only a sign of cell senescence [16]. Cellular senescence, which refers to irreversible cell growth arrest [17], is another issue related to hBM-MSC cultivation. It limits the proliferative capacity of primary cells in culture [18], impairs therapeutic potential of hBM-MSC [19], and increases the risk of cell neoplastic transformation [20, 21]. Although some publications reporting the alarming finding of malignant transformation of hMSCs [22], including hBM-MSCs [23], later on have been retracted [24, 25], there is still debate concerning the genetic stability of hMSCs and the implication for clinical safety [26, 27].

It is of great scientific interest to investigate MSCs isolated from low human bone marrow volume for potential medical use. Recently, our group has showed that MSCs can be successfully isolated by red blood cell lysis method from residual bone marrow transplantation material and expanded *in vitro* to clinically relevant numbers [28]. The aim of this study was therefore to assess hBM-MSC immunophenotype as proposed by The International Society for Cellular Therapy (ISCT) [29]; to evaluate proliferative capacity, senescence status and cytogenetic stability, as determined by The European Medicine Agency (EMA) [30]; and to apply array technology as suggested by the U.S. Food and Drug Administration (FDA) [31]. Our results highlight the identity, proliferation capacity, and genomic safety of MSCs isolated from low human bone marrow volume and reveal 38 new hBM-MSCs potential senescence markers during prolonged cultivation *in vitro*.

RESULTS

Morphology

We observed hBM-MSCs microscopically at every passage. Adherent long spindle-shaped or flat fibroblast-like cells were detected 24-48 hours after isolation. Such morphology retained up to passages 3-4 (P3-P4) (Figure 1A). Later on the proportion of enlarged cells with altered morphology gradually increased, which became obvious at late passages 6-7 (P6-P7) (Figure 1B). An average spread cell area was significantly enlarged at late passages of individual samples (Figure 1C).
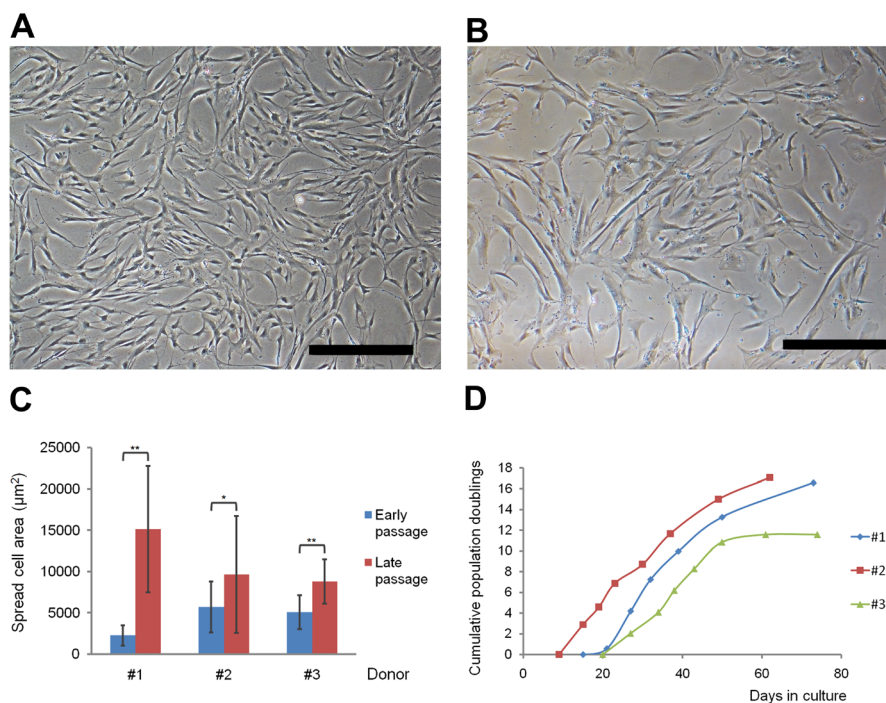
Figure 1: Morphology and proliferation kinetics of hBM-MSCs during *in vitro* culture. Typical homogeneous population of fibroblast-like cells at P4 **A.** and heterogenous population including enlarged cells with altered morphology at P7 **B.** Original magnification x40, scale bars represent 500 μm. **C.** Average spread cell area at early (P3-P4) and late (P6-P7) passages of individual samples (*$P < 0.05$; **$P < 0.001$). **D.** Growth kinetics of MSC cultures from 3 donors scored as cumulative population doublings (y-axis) plotted against time in culture (x-axis). Each marker represents a passage.
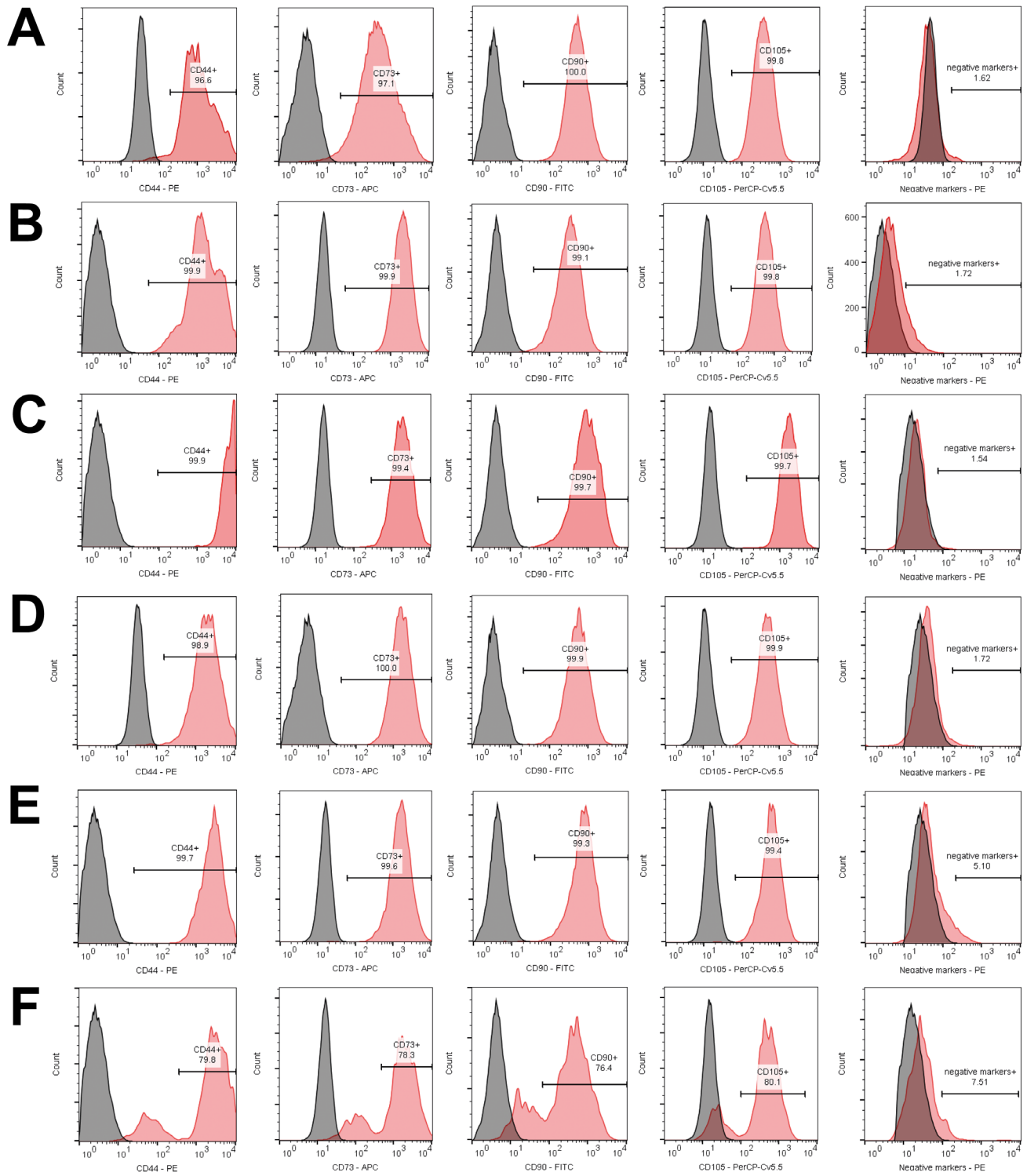
Figure 2: Immunophenotype of hBM-MSCs in long term culture. Analysis of samples #1 **A.**, #2 **B.**, #3 **C.** at the early passages and analysis of samples #1 **D.**, #2 **E.**, #3 **F.** at the late passages is demonstrated. Histograms on the left (grey) represent unstained cells, and histograms on the right (red) represent stained cells.

Proliferation

MSCs showed a slower proliferation after isolation and reached P1 after 21±6 days (Figure 1D). From P1 to P3 (sample #1) or P4 (samples #2 and #3) the cells proliferated faster and CPDs resulted in 8.08±0.74 after additional 14.00±2.65 days. In late culture the gradual slow-down in the cellular growth occurred and it took 34.67±5.51 more days to complete with 15.08±3.04 CPDs.

Flow cytometry analysis

In the early passages over 99% of the cells were positive for CD73, CD90, and CD105, while below 2% of the cells expressed CD11b, CD19, CD45, CD34, and HLA-DR (Figure 2A-2C). However, part of MSC population of #3 sample lost the expression of positive markers and gained the expression of negative markers in P7 (Figure 2F). The expression of negative markers also increased in #2 sample in P7, although expression of positive markers remained stable (Figure 2E). The immunophenotype of #1 sample in P6 did not change (Figure 2D). Mean viability of hBM-MSCs was 94.02±2.92% at early passages and 93.47±5.61% at late passages. The side-scatter (SSC) was 337.00±55.44 units at early passages and 391.67±27.00 units at late passages, although the difference was not statistically significant (P = 0.085).

Senescence-associated β-galactosidase staining

The cell dyeing for SA-β-gal showed that long-term culture is accompanied by increase in senescent cells. There were 1.59±0.94% SA-β-gal-positive cells at P3-P4 and 41.97±4.57% at P6-P7 (P = 0.0043) (Figure 3A-3B).

Karyotype

To investigate the effects of long-term culture on genomic integrity, we analyzed the karyotype by G-banding at P3-P4 and P6-P7 (Figure 4A-4B). Nearly 87% of the cells at early passages and nearly 88% of the cells at late passages had normal diploid karyotype (2n, n = 23). No clonal numerical or structural cytogenetic alterations were observed. We detected random aneuploidies at early passages 45,-10; 47,+15; 47,+22 (sample #1); 44,-20,-21 and 45,-22 (sample #2); and 47,+22 (sample #3). At late passages we detected 44,-X,-20 (sample #1) and 45,-20 (sample #2) (data not shown). None of these abnormalities was considered clonal because they all were seen in a single cell per specimen, most likely, due to technical preparation of chromosomes.

Gene expression

To further evaluate the hBM-MSCs, we measured the expression of 162 different genes related to stemness, mesenchymal stem cells and cell senescence using commercial qPCR arrays at P3-P4 and P6-P7. Altogether, the expression of 154 genes was detected (C_t < 33) in early passage MSCs and the expression of 156 genes was detected in late passage MSCs. From 162 genes, 4 genes were significantly (P < 0.05) up-regulated (≥2 fold) and 9 genes were significantly down-regulated in late passage hBM-MSCs when compared with early passage MSCs (Figure 5A-5B, Table 1). This represents 8.02% of all genes investigated in the study. In order to better understand the underlying biological processes in late passage BM-MSCs, we performed gene enrichment analysis of set of 13 genes with significantly altered expression (Table 2).

A

B
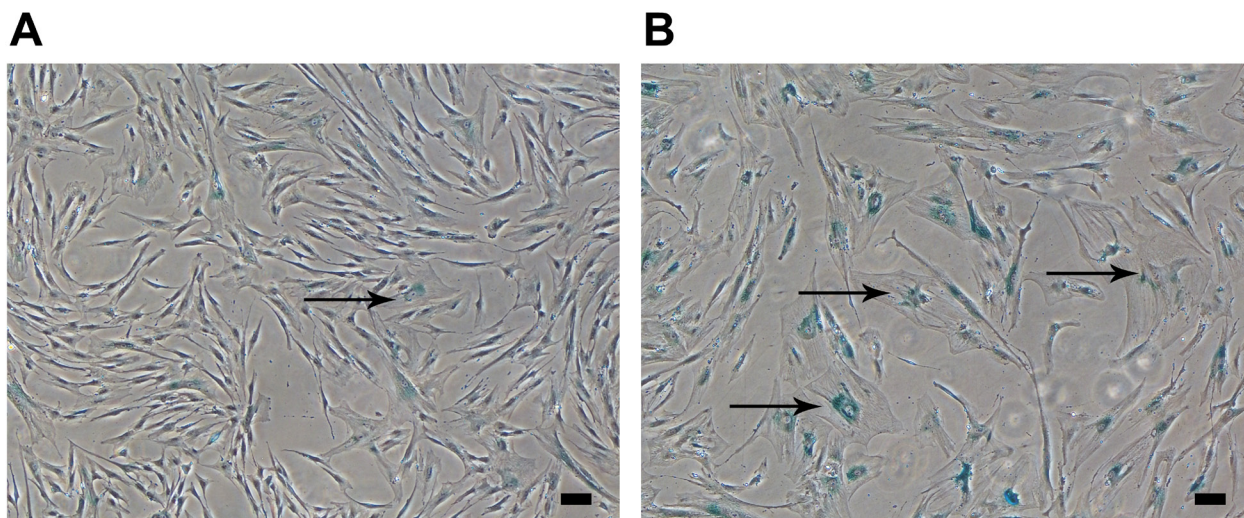
Figure 3: hBM-MSC senescence during *in vitro* culture. Representative images of enlarged with altered morphology SA-β-gal-positive cells (indicated with black arrows) at P4 **A.** and P7 **B.** Original magnification x40, scale bars represent 100 μm.

Table 1: Significant fold regulation of genes and miRNAs in late passage versus early passage hBM-MSCs

| Gene/miRNA Symbol	Fold Regulation	*P*-value	miRNA Symbol	Fold Regulation	*P*-value
ACTA2	3.1249	0.017625	*hsa-miR-935*	-4.4097	0.038848
POU5F1	2.9597	0.026534	*hsa-miR-193a-3p*	-3.5535	0.000240
PTPRC	2.5796	0.015727	*hsa-miR-200a-3p*	-2.8797	0.038458
THBS1	2.0582	0.031849	*hsa-miR-187-3p*	-2.6816	0.008664
E2F3	-6.7673	0.040950	*hsa-miR-192-5p*	-2.6692	0.000444
CCNB1	-3.6560	0.016672	*hsa-miR-130b-5p*	-2.6437	0.002407
CHEK1	-3.3642	0.014370	*hsa-miR-218-5p*	-2.6433	0.002247
PLAU	-2.7200	0.000930	*hsa-miR-92a-1-5p*	-2.6194	0.034756
TBX2	-2.5941	0.009478	*hsa-miR-877-5p*	-2.5503	0.012980
TBX3	-2.2662	0.012653	*hsa-miR-337-3p*	-2.5181	0.002904
CDC25C	-2.2195	0.037200	*hsa-miR-106b-3p*	-2.3068	0.021249
E2F1	-2.1738	0.030206	*hsa-miR-139-5p*	-2.3091	0.001997
PCNA	-2.0236	0.044944	*hsa-miR-455-5p*	-2.2997	0.001550
hsa-miR-422a	5.7364	0.016040	*hsa-miR-188-3p*	-2.2305	0.015998
hsa-miR-376b-3p	5.6578	0.000018	*hsa-miR-875-3p*	-2.1947	0.009676
hsa-miR-200a-5p	5.0577	0.003929	*hsa-miR-224-5p*	-2.1874	0.001306
hsa-miR-219a-2-3p	4.0963	0.036284	*hsa-miR-29a-5p*	-2.1573	0.043583
hsa-miR-639	3.8552	0.006863	*hsa-miR-25-5p*	-2.1203	0.046255
hsa-miR-223-3p	3.7575	0.049123	*hsa-miR-660-5p*	-2.1088	0.001035
hsa-miR-608	3.6014	0.002329	*hsa-miR-576-5p*	-2.0967	0.002239
hsa-miR-429	2.7041	0.013235	*hsa-miR-15b-3p*	-2.0742	0.031753
has-miR-210-3p	2.1681	0.019652	*hsa-miR-29b-1-5p*	-2.0528	0.006955
hsa-miR-335-5p	2.0328	0.028915	*hsa-miR-7-5p*	-2.0189	0.045330

miRNA expression

The miRNA profiles of hBM-MSCs from early and late passages were analyzed with the commercial qPCR-based array for human miRNA. Overall, the expression of 358 miRNAs was detected (C_t < 33) in early passage hBM-MSCs and the expression of 365 miRNAs was detected in late passage cells. Analysis showed significant (P < 0.05) ≥2 fold changes in expression of 33 of 420 miRNAs (Figure 5C, Table 1), and these constituted 7.86% of all evaluated miRNAs.

DISCUSSION

In this study we isolated MSCs from residual human bone marrow transplantation material, as described earlier [28], expanded *in vitro* to clinically relevant
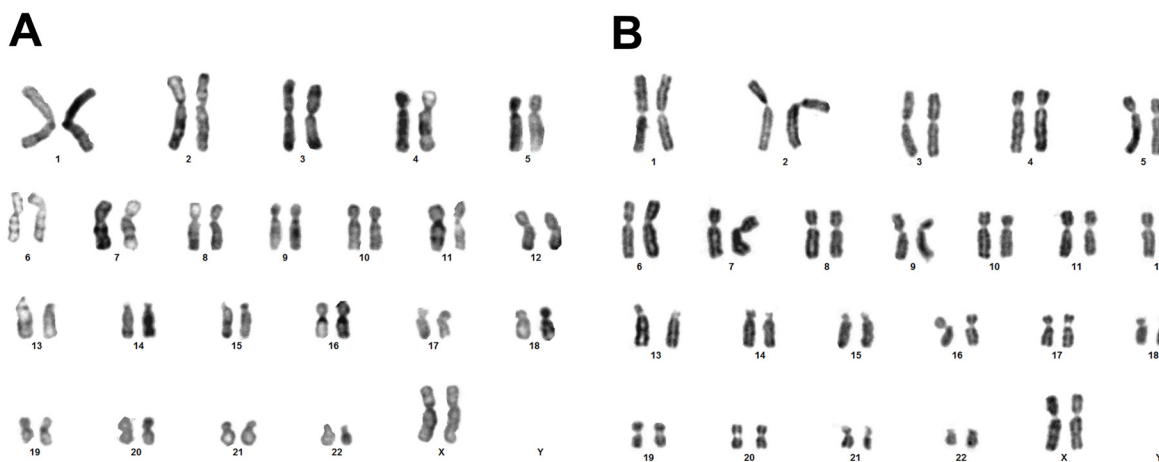
Figure 4: hBM-MSC karyotype during *in vitro* culture. Representative karyograms of hBM-MSC normal diploid karyotype (2n, *n* = 23) at P3 **A.** and P6 **B.**

Table 2: Gene Onthology (GO) term* enrichment analysis of set of 13 genes with altered expression in late passage hBM-MSCs

Gene Onthology category	Biological process	Sample frequency	Background frequency	P-value
GO:0050896	response to stimulus	13	7510	1.34E-02
GO:0060255	regulation of macromolecule metabolic process	12	5396	6.93E-03
GO:0051716	cellular response to stimulus	12	6121	3.01E-02
GO:0006950	response to stress	11	3492	1.26E-03
GO:0009893	positive regulation of metabolic process	10	3384	1.74E-02
GO:0009059	macromolecule biosynthetic process	10	3614	3.24E-02
GO:0010604	positive regulation of macromolecule metabolic process	10	2579	1.30E-03
GO:0048513	organ development	9	2728	3.76E-02
GO:0031325	positive regulation of cellular metabolic process	9	2750	4.03E-02
GO:0042127	regulation of cell proliferation	9	1438	1.51E-04
GO:0033554	cellular response to stress	8	1630	9.66E-03
GO:0007049	cell cycle	8	1290	1.61E-03
	Cellular function			
GO:0005667	transcription factor complex	4	291	2.82E-02

*Gene Ontology (GO) terms were used to define the biological processes, cellular and molecular functions using the Gene Ontology Consortium. Background frequency is the number of genes annotated to a GO term in the entire *H. sapiens* background set, while sample frequency is the number of genes annotated to that GO term in the input list. The terms listed in the table are the most frequently annotated with $P < 0.05$. No genes were statistically significantly annotated to GO terms for molecular functions.

numbers and characterized these cells by evaluating adherence to plastic, morphology, proliferative capacity, immunophenotype, senescence status, karyotype stability, gene and miRNA expression profiling, as proposed by ISCT [29], EMA [30], and FDA [31]. hBM-MSC lifespan was categorized as early passage (P3-P4) and late passage (P6-P7) according to proliferation ability and the percentage of SA-β-gal, similarly as proposed before [32].

Proliferation is a fundamental property of stem cells necessary for self-renewal and expansion and defining stem cell degree of stemness [33]. Population doubling (PD) is a precise way to measure cell growth [34] and is
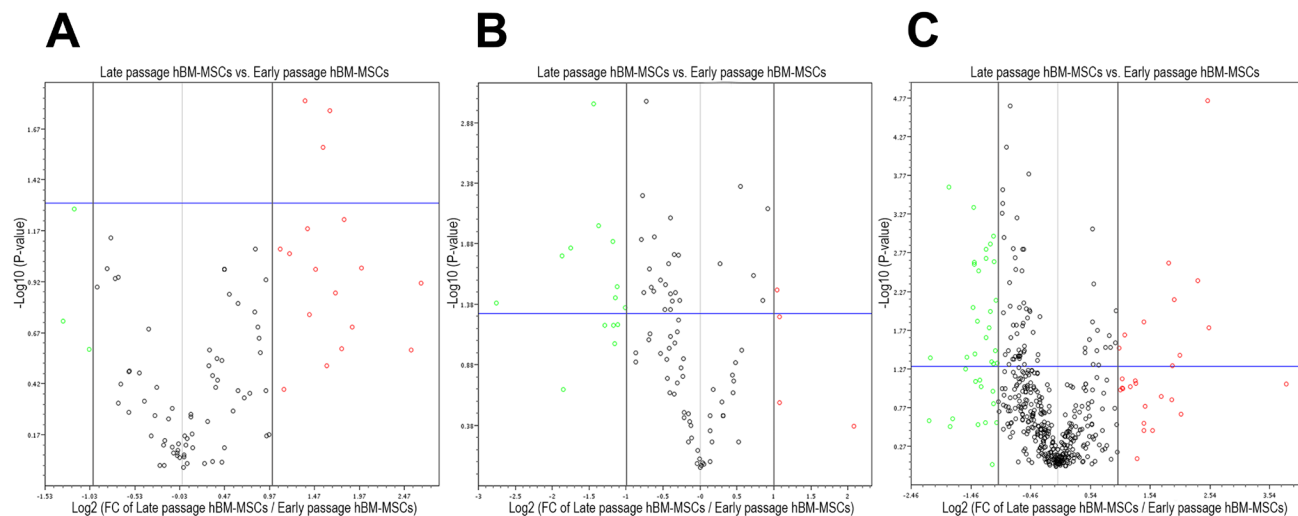
Figure 5: Volcano plots of human mesenchymal stem cell related gene A., cellular senescence related gene B. and miRNA C. expression changes in late passage versus early passage hBM-MSCs. Red dots are the genes whose expression increased more than 2 fold, while green dots are decreased more than 2 fold. Vertical grey side-lines represent fold-change cutoff (≥2 fold) and horizontal blue line represents *p*-value cutoff ($P < 0.05$). Genes and miRNAs whose fold expression changes and *P*-values exceeded the boundaries are listed in the Table 1.

recommended by the Cell Products Working Party (EMA) to describe the time for cells in culture [35]. We showed that hBM-MSCs at early passages are highly proliferative. Cryopreserved BM-MSCs at P1 can be expanded to high clinically relevant yield of cells within two weeks at P3-P4 with CPDs 8.08±0.74 (Figure 1D), which would result in hundreds of millions of cells. MSCs at early passages maintained a spindle-shaped or fibroblast-like morphology (Figure 1A), typical for adult hBM-MSCs [36], were adherent and exhibited immunophenotype (Figure 3A-3C) in accordance with ISCT guidelines [29].

Genomic instability of MSCs is one of important concern for clinical use of MSCs [37] because it enables the cells to acquire tumor cell characteristics [11]. Therefore the cytogenetic analysis is essential for verifying the safety of MSCs [38] since the maintenance of a normal karyotype is a reliable indicator of genetic stability of MSCs [39]. By conventional karyotyping of cultured hBM-MSCs using G-banding, which is still a gold standard of all cytogenetic techniques [40], we showed that BM-MSCs at P3-P4 had a normal karyotype and none of the samples had clonal aberrations (Figure 4A). These results indicate that the genomic stability of our MSCs would not prevent their potential use in a clinical application, similarly as shown earlier [15].

By expanding hBM-MSCs using additional three passages (until P6-P7) we investigated the possibility to achieve additional clinically relevant amounts of cells. However, in the late passages the hBM-MSCs growth gradually decreased (Figure 1D) and cells acquired an enlarged flattened morphology (Figure 1B-1C), indicating MSC senescence [41]. Cellular senescence is defined as irreversible cell cycle arrest [17]. By staining cells for SA-β-galactosidase, the most widely used biomarker for senescent cells [42], we confirmed that after 15.08±3.04 PDs (Figure 1D) almost half of late passage hBM-MSCs reached senescence, whereas only a few cells stained positively in the early passages (Figure 3A-3B).

Interestingly, the onset of senescence in long-term culture manifested differently on hBM-MSC immunophenotype in each sample. Surface marker expression of #1 sample remained stable throughout the *in vitro* expansion (Figure 2D), in agreement with Dmitrieva et al. [43] and Somasundaram et al. studies [44]. Dmitrieva et al. demonstrated that hBM-MSC enter senescence after P3-P4, but the cells were CD105/CD90/CD166/CD73 positive and negative for CD34, CD19, CD14 and CD45 at all passages. Somasundaram et al. revealed remarkable (>90%) expression of CD73, CD90, and CD105 and sparse (< 10%) expression of CD34, CD45, and HLA-DR of hBM-MSC irrespective of extensive culturing when the majority of samples lost potential to grow beyond P15. However, the expression of negative markers increased up to 5.10% in #2 sample in P7, although expression of positive markers remained stable (Figure 2E). Moreover, part of non-proliferating MSC population of #3 sample

lost the expression of positive markers and gained the expression of negative markers in P7 (Figure 2F). Wagner et al. [45] has demonstrated that *in vitro* expansion has a major impact on the level of surface marker expression of human BM-MSCs. Surface antigen detection was much higher in early passages when compared to senescent passages. However, quantification (%) of expression was not presented in that study. Our results were unexpected and indicate that identification of late passage senescent MSCs by using cell-surface markers can be complicated. Therefore possible changes in standard surface marker expression during prolonged *in vitro* expansion require further investigations. We also revealed long-term culture-related, however not statistically significant, differences in cell granularity, another hBM-MSC senescence associated feature [46]. Interestingly, the karyotype of late passage senescent cells remained stable (Figure 4B), compatible with data obtained on long-term expanded hBM-MSCs by other groups [5, 14] and opposing to the recent finding that senescence-prone human MSCs are highly aneuploid [47].

To date no molecular markers are available, which specifically reflect the degree of cellular aging in a population of MSCs [48]. Molecular analysis of a suitable panel of genes might provide a powerful tool to track cellular aging of MSCs and thus to assess efficiency and safety of long-term expansion [42]. Real-time quantitative PCR is the gold-standard technique for gene expression measurements [49], therefore we investigated the cells using qPCR arrays. Transcriptome analysis of 162 different genes revealed 4 significantly ($P < 0.05$) up-regulated (≥ 2 fold) genes and 9 significantly down-regulated genes in P6-P7 hBM-MSCs when compared to P3-P4 cells (Table 1). *Pou5f1* (Oct4) is a critical regulator of pluripotency in embryonic stem cells and might be reactivated in response to culture conditions [50]. Exogenous OCT4 overexpression has been shown to induce early senescence of hBM-MSCs [51], virtually consistently with our observations. *PTPRC* encodes the protein tyrosine phosphatase CD45 not characteristic for hMSCs [29] and its overexpression decreases cytokine-induced signaling [52]. *ACTA2*, which was the most upregulated in our study, codes a smooth muscle α actin isoform enabling hBM-MSCs to contract the extracellular matrix (ECM) components [53]. *THBS1* codes thrombospondin-1, which is secreted and incorporated into ECM [54]. We determined *THBS1* upregulation in senescent hBM-MSCs in concordance with Yoo et al. report [55]. *PLAU* gene encodes enzyme urokinase-type plasminogen activator (uPA), which regulates ECM degradation, cell adhesion, and inflammatory cell activation [56] and which activity depends on cytoskeleton reorganization [57]. An impairment of cytoskeleton remodeling and/or organization has been associated with hBM-MSC senescence [58]. E2F1 and E2F3 control the expression of numerous genes involved in DNA replication and cell cycle progression. Deregulation of these transcription

factors results in the induction of senescence [59], with the loss of E2F3, which was the most downregulated in this study, having the most pronounced effect [60]. *TBX2* and *TBX3* encode T-box proteins that function as transcriptional repressors [61]. Inhibition of both leads to cell senescence [62]. In contrast to our findings, Choi et al. showed higher TBX2 expression in late passage senescent hBM-MSCs [63]. Chk1 protein kinase is essential for the human G2 DNA damage checkpoint[64] and has been shown to be downregulated in senescent hBM-MSC [65]. *PCNA* codes proliferating cell nuclear antigen expressed exclusively in actively proliferating cells [66]. E2F1-3 induces expression of PCNA [67], which is regulated by Chk1[68]. We showed *PCNA* repression in late passage senescent hBM-MSCs, in compliance with Choi et al. report [63]. Human Cdc25C phosphatase is a key activator of the cyclin B1/Cdk1 complex [69], which is essential for entry into mitosis [70]. CDC25C inhibition promotes cell cycle arrest [71], and G2/M arrest is characteristic for stress-induced premature senescence [72]. We showed *CCNB1* downregulation in senescent hBM-MSCs, in agreement with Noh et al. study [65]. Functional gene ontology analysis revealed that these genes are associated with biological processes as cell cycle, metabolism, cell aging, and response to stress (Table 2), all of which are important causes of cellular senescence [73]. In sum, these results together with literature data strongly suggest that identified 13 genes are interconnectedly related to hBM-MSC premature senescence. On the other hand, to our knowledge, we for the first time show that the expression of *POU5F1, PTPRC, ACTA2, E2F1, E2F3, Tbx3, PLAU* and *CDC25C* genes is altered in senescent hBM-MSCs during long-term expansion *in vitro*.

PCR array data were deposited into a public database Gene Expression Omnibus (http://www.ncbi.nlm. nih.gov/geo/) under accession number GSE68933.

The p53/p21/Rb and p16/RB axes are key signaling pathways involved in the induction of cell senescence [74]. In particular, RB and its family members, p107 and p130, are essential for the onset of senescence cell cycle arrest [17]. An unexpected finding of this study was the constant (within 2 fold) expression and/or nonsignificant changes ($P{\geq}0.05$) of the expression of these crucial genes (data available in the database GEO under accession number GSE68933). These results stand out from contrary data obtained by other laboratories. Cheng et al. demonstrated that *p16*, *p21*, and *p53* are significantly upregulated in senescent hBM-MSCs [75]. Shibata et al. showed significant increase in the expression level of *p16* and no significant changes in the expression of *p21* and *p53* at the end of hBM-MSC life span [76], similar results are reported by Tarte with colleagues [16]. Kim et al. showed unaltered *p16* expression and reduced expression of *p53* during long-term culturing of hBM-MSCs [14]. However, we cannot rule out the possibility that genes exhibiting a less than twofold change may be of biologic value [77].

While the functions of RB1, p107 and p130 in the biology of MSCs remain largely uncharacterized [78].

MicroRNAs, also called miRNAs, are small 19-22 nucleotide sequences of noncoding RNA that work as endogenous epigenetic key gene expression regulators [79]. Only recently senescence-associated miRNAs (SA-miRNAs) have emerged as important effectors of senescence [80]. Therefore we were particularly interested in the possible involvement of the miRNAs in the regulation of hBM-MSC senescence. By using miRNA qPCR array, we identified 33 miRNAs with altered expression in late passage senescent hBM-MSCs (Table 1). Among the top downregulated, *miR-935* previously has been shown to be downregulated in elder hBM-MSCs [81]; *miR-193a* has been reported to regulate uPA [82], to target oxidative stress pathway [83], and not to be repressed in normal BM cells [84]. Additionally, *miR-337-5p* was shown to be differentially expressed in pediatric hBM-MSCs comparing to adult hBM-MSCs [85]. Yoo et al. demonstrated that *miR-29b* is downregulated in senescent hBM-MSC compared to young hBM-MSCs, but *miR-455-3p*, unlike in our study, was upregulated [86]. Among the most upregulated, *miR-376b* has been shown to be differentially expressed in pediatric hBM-MSCs when comparing to adult hBM-MSCs [85]; *miR-200a* has been associated with the oxidative stress [87] and shown to be activated in stress-induced senescent cells [88]. Tome et al. demonstrated *miR-335* increase in hBM-MSC *ex vivo* culture and correlation with cell senescence [89], similarly to our data. Together, these results along with other reports further firmly propose that hBM-MSCs underwent *in vitro* culture induced premature senescence. Besides, as far as we know, our report is the first to link the change of expression of new 30 miRNAs to hBM-MSC senescence during prolonged *in vitro* expansion.

Recently, Balakrishnan with colleagues determined that *miR-193a* and *miR-200a* of hBM-MSCs regulate hematopoietic stem cell niche-defining genes [90]. We demonstrated that the expression of these miRNAs, surprisingly, is one of the most altered in senescent hBM-MSCs (Table 1). Interestingly, senescence phenotype of the hematological patient hBM-MSCs only very lately has been revealed [91-96].

Taking everything into account, we state that MSCs isolated from residual bone marrow transplantation material and expanded to clinically relevant numbers are genomically stable and retain identity and high proliferation capacity. It is a crucial requisite for clinical application in terms of donor comfort and recipient safety. However, the cells enter senescence state after long-term expansion, most likely, due to culture-induced stress. We propose that the identified novel hBM-MSC senescence associated genes and miRNAs provide a better understanding of the mechanisms involved in hBM-MSC aging, significantly contributing to basic science and cellular therapy quality control development and revealing

new clues of hematological disease processes for future investigations. Further larger research in this area is needed to validate the claims of this study.

MATERIALS AND METHODS

Bone marrow collection

Bone marrow (BM) specimens were collected from healthy adult donors after obtaining written informed consent at Vilnius University Santariskiu Clinic, Children Hematology and Transplantation Center. The study was reviewed by Vilnius Regional Committee of Biomedical Research, Lithuania (Permission No 158200-09-381-104).

Isolation of MSCs

MSCs were isolated from 3 donors (#1 female, age 24; #2 male, age 38; #3 female, age 28) using red blood cell lysis method as described earlier [28]. Briefly, BM samples were mixed with erythrocyte lysis buffer (Qiagen, Germany) and centrifuged for 5 min at 480g. After removal of the supernatant, the pellet was resuspended with 5 ml of RPMI 1640 medium (Invitrogen, UK) and washed twice through centrifugation. Finally, all amount of resuspended cell suspension was placed into T75 cm^2 tissue culture flask (BD Biosciences, France) and allowed to adhere for 24 hours in the DMEM medium containing 10% of fetal bovine serum (FBS) (StemCell Technologies, Canada) and 1% penicillin/streptomycin (Gibco, USA) at 37°C with 5% CO_2 and fully humidified atmosphere.

Culture of MSCs

After 24 hours the medium was removed and the cells were washed with phosphate buffered saline (PBS). Human MesenCult MSC Basal Medium containing 10% of MesenCult FBS for human MSCs (StemCell Technologies, Canada) and 1% penicillin/streptomycin (Gibco, USA) was used for subsequent cultivation of MSCs. The medium was changed every 3-4 days. When cells reached 80-90% confluence, they were harvested with 0.25% trypsin-EDTA (Invitrogen, UK), counted and subcultured with seeding density 4000/cm^2 into new T75 cm2 flasks under the standard conditions.

Morphology

Cell morphology was determined using Nicon inverted phase contrast microscope (models T*i*-S and TS100) and NIS-Elements imaging software (version 3.22.00). For spread cell area analysis nineteen cells from each image were chosen at random and manually outlined.

Individual cell areas were measured. Image analysis was performed in ImageJ v1.50e image processing tool set.

Cryopreservation and thawing

MSCs were cryopreserved at P1 and P3-P4. Cells were mixed with MSC Freezing Solution (Biological Industries, Israel) and placed into Mr. Frosty Freezing Container (Thermo Scientific, USA) in -80°C freezer for -1°C/minute freezing rate. After 24 hours cryovials were transferred to -150°C freezer for storage. After six months of storing samples were rapidly thawed by placing cryovials in a water bath at 37°C and diluted in a slow dropwise manner with pre-warmed fresh culture medium. After centrifugation at 150 g for 10 min, MSCs were plated with seeding density 4000/cm^2 into T75 cm^2 flasks and incubated under the standard conditions.

Cell number and proliferation kinetics

Cell number was determined using a CASY cell counter and analyzer (Roche, Germany) at each passage and long-term growth kinetics *in vitro* was assessed by determining cumulative population doublings (CPDs). The number of population doublings (PDs) was calculated using the formula: PD = $\log(X_b/X_a)\cdot3.3$, where X_a represents the initial cell number, X_b represents the cell harvest number, and 3.3 is a coefficient. PD of each passage was calculated and added to the PD of the previous passage level to obtain the CPD.

Flow cytometry

hBM-MSCs were characterized at P3-P4 and P6-P7 by flow cytometry using antibodies to CD44, CD73, CD90, and CD105 cell surface markers and using a mixture of antibodies to CD34, CD11b, CD19, CD45, HLA-DR cell surface markers (BD Stemflow™ Human MSC Analysis Kit). After harvesting, the cells were washed with PBS and treated according to the manufacturer's protocol. Viability of the MSCs samples was assessed by 5-minute 7-AAD staining and cell granularity was determined by side-scattered (SSC) light evaluation. Cytometric measurements were performed on BD LSR II flow cytometer. 10.000 cells per tube were analyzed with FlowJo X software.

Senescence-associated β-galactosidase staining

Senescence of cultivated hBM-MSCs at passages 3-4 and 6-7 was studied using Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, Germany) according to the manufacturer's protocol. At the end of staining procedure, ten pictures were taken from random

areas of each culture. The percentage of senescent cells was calculated using the following formula: (number of cells with intracellular blue deposits/ total number of cells) x 100%.

Karyotype analysis

Cytogenetic evaluation by G-banding method was conducted on hBM-MSCs at passages 3-4 and 6-7. Colchicine was added into each culture at a final concentration of 0.2 μg/ml for 4 hours at 37°C. MSCs were harvested using trypsin and resuspended in a hypotonic 0.075 mM KCl solution for 30 min at 37°C. After centrifugation the cells were fixed with methanol:acetic acid (3:1) solution. After dropping the cell suspension onto microscope slides, these were trypsinized and stained with Giemsa solution (Sigma-Aldrich, Germany). Slides were scanned, metaphases we captured and analyzed with Leica CytoVision® (USA) platform. 7 to 17 metaphase spreads were analyzed for chromosome number and structure abnormalities at each established passage. Karyotypes were described following the recommendations of the International System for Human Cytogenetic Nomenclature 2013 [97].

RNA isolation

Total RNA was isolated from hBM-MSCs using miRNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Total RNA concentration and quality were checked using a NanoDrop spectrophotometer and verified by analysis on an Agilent 2100 Bioanalyzer using RNA 6000 Nano LabChip (Agilent Technologies, USA).

PCR arrays

hBM-MSC samples of P3-P4 and P6-P7 were analyzed using Human Mesenchymal Stem Cell RT² Profiler™ PCR Array (PAHS-082Z, SABiosciences, Qiagen) and Human Cellular Senescence RT² Profiler™ PCR Array (PAHS-050Z). Template cDNA was synthesized from 800 ng of the total RNA using the RT² First Strand Kit (Qiagen) following manufacturer's protocol. The reaction mix was prepared by mixing cDNA with 2x RT² SYBR Green ROX FAST Mastermix (Qiagen) and 20 ml of the cocktail was aliquoted into each well on the PCR array. Each array consisted of a panel of 96 primer sets of 84 mesenchymal stem cell or cellular senescence genes, 5 housekeeping genes, and 7 quality controls. PCR arrays were performed in Rotor-Gene Q thermocycler (Qiagen), as follows: 95°C, 10 sec for initial denaturation, and 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Each sample was tested in technical duplicate. The data were analyzed using web-based RT² Profiler PCR Array Data Analysis v3.5 software. The fold-change in target gene expression was calculated using the $\Delta\Delta C_t$ method and normalized to the geometric mean of 5 housekeeping genes (ACTB, B2M, GAPDH, HPRT1, and RPLP0) according to SABioscience guide. A more than two-fold change in gene expression was considered as the up- or down-regulation of a specific gene expression. Differences were considered significant when P value < 0.05.

miRNA PCR array

The miRNA levels in hBM-MSCs of early and late passages were analyzed with miRNome miScript miRNA PCR Array (MIHS-216ZR-4, SABiosciences, Qiagen). Template cDNA was synthesized from 600 ng of the total RNA with miScript II RT Kit using miScript HiSpec Buffer (Qiagen) following manufacturer's protocol. The templates were mixed with RT² SYBR Green qPCR Master Mix (Qiagen) and 20 μl aliquoted into each well of 5 PCR arrays. Each array consisted of a panel of 96 primer sets of 84 miRNAs of interest, 2 *C. elegans* miR-39, and 10 controls. PCR was performed in Rotor-Gene Q thermocycler (Qiagen), as follows: 15 min at 95°C and 40 cycles of 15 sec at 94°C, 30 sec at 55°C, and 30 sec at 70°C. Each sample was tested in technical duplicate. The miRNA data were analyzed using online software miScript miRNA PCR Array Data Analysis. The relative expression of each target miRNA was determined with the $\Delta\Delta Ct$ method and normalized to the geometric mean of 6 small RNAs (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6-2) according to SABioscience guide. A miRNA was considered differentially expressed if it showed more than two-fold change and P value < 0.05 indicated significance.

Gene ontology analysis

Gene Ontology Consortium (http://geneontology.org/) was used for enrichment analysis of specific gene sets[98]. Genes were classified to gene ontology (GO) terms in three categories: molecular function, cellular component and biological process.

Data analysis

Statistical analysis was performed using SPSS software (version 21). The Student's paired t-test was performed to assess statistical differences which were considered significant when P value < 0.05.

Data access

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CONFLICTS OF INTEREST

The authors declare no competing interests.

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