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LETTER TO THE EDITOR

Overexpression of CDKN2B (p15INK4B) and altered global DNA methylation status in mesenchymal stem cells of high-risk myelodysplastic syndromes

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Myelodysplastic syndromes (MDSs) are a heterogeneous group of clonal hematopoietic stem cell (HSC) malignancies that are characterized by ineffective bone marrow hematopoiesis, peripheral blood cytopenias and a substantial risk of progression to acute myeloid leukemia.[1–3] There is evidence that alterations of the direct interaction between HSCs and stroma contribute to abnormal HSC growth and maturation.[4]

Mesenchymal stem cells (MSCs) have been defined as non-hematopoietic cells that primarily reside in bone marrow (BM) and are capable of producing different cell lineages. They are important components of the hematopoietic microenvironment and are important in supporting and regulating the proliferation and differentiation of hematopoietic stem cells, as well as in immunoregulatory functions.[5]

We characterized the molecular and functional properties of BM-derived MSCs isolated from 24 high-risk IPSS MDS patients and compared them with 10 older healthy donors (5 ml, median age 79 years, range 60–82 years) and 10 younger healthy donors (5 ml, median age 17 years, range 16–45 years).

Clinical data and BM samples were obtained from the local MDS registry with informed consent from each patient. This protocol was approved by the Ethics Committee and followed the Declaration of Helsinki guidelines. Healthy BM was harvested from BM donors or from patients undergoing hip surgery after obtaining written informed consent from all participants or from parents or guardians of those under 18 years of age. Their clinical and biological characteristics are shown in Table 1.

After cellular adhesion in *in vitro* culture, MDS-MSCs displayed fibroblast-like shapes under a phase–contrast microscope; however, since the beginning of the culture they exhibited an altered morphology and appeared larger, flattened and granular.

Using flow cytometry we compared the expression of molecules that have important roles in cell adhesion, such as CD90, CD44 and CD29, and typical mesenchymal markers, such as CD105 and CD73. The MDS-MSCs and the healthy donor MSCs exhibited the same phenotype and were positively stained for the antigens listed above. They were negative for hematopoietic and endothelial markers CD34, CD45, CD14 and CD31.

MDS-MSCs showed reduced proliferative capacity compared with controls (expansion potential of MDS-MSCs vs old donors: 0.88 ± 1.1 logs, 2.5 ± 3.5 population doublings (PDs) vs 2.93 ± 0.64 logs, 9.5 ± 2.3 population doublings) ($P < 0.05$). Moreover, 40% of the samples could not be expanded *in vitro* (Figure 1A). This result

Table 1. Clinical and laboratory data of patients studied

No.	*Age/gender*	*FAB*	*WHO*	*% Blasts*	*Karyotype*	*IPSS*	*r-IPSS*
1	69/M	RAEB	RAEB-1	10	del7 (15%)	1.5 INT2	5 INT
2	68/M	RAEB	RAEB-2	15	Normal	2 INT2	6.5 VH
3	77/M	RAEB	RAEB-2	15	Not evaluable		
4	73/M	RAEB-T	AML	25	Normal	2.5 HIGH	7 VH
5	56/M	RAEB	RAEB-2	12	tris1q, mon7q (25%)	2.5 HIGH	6 VH
6	82/F	RAEB	RAEB-2	15	del5, tris1, tris11, adm14	3 HIGH	10 VH
7	72/M	RAEB-T	AML	28	Normal	2.5 HIGH	6.5 VH
8	79/M	RAEB-T	AML	30	Not evaluable		
9	84/M	RAEB	RAEB-2	20	Normal	2 INT2	6.5 VH
10	77/F	RAEB-T	AML	28	Normal	2.5 HIGH	5 INT
11	74/F	RAEB	RAEB-2	20	Normal	2 INT2	8 VH
12	71/M	RAEB-T	AML	30	Not evaluable		
13	69/M	RAEB	RAEB-2	15	ct5, del5q, del7q, t(12;13)(12p;13q) (85%)	2.5 HIGH	9 VH
14	80/F	RAEB	RAEB-2	11	Normal	2 INT2	8 VH
15	62/F	RAEB	RAEB-2	13	mon7	2.5 HIGH	7 VH
16	61/M	RAEB	RAEB-2	15	del5q, hyperdiploide	2 INT2	6 HIGH
17	75/M	RAEB	RAEB-2	15	Not evaluable		
18	66/M	RAEB	RAEB-2	12	tris11 (7%)	2 INT2	6 HIGH
19	64/F	RAEB	RAEB-2	15	tris1q (50%)	2.5 HIGH	7.5 VH
20	82/F	RAEB	RAEB-2	15	del5q (35%)	1.5 INT2	5.5 HIGH
21	60/M	RAEB	RAEB-2	20	Normal	2 INT2	8 VH
22	77/M	RAEB-T	RAEB-2	30	del5q, del12p, del	3.5 HIGH	9 VH
23	79/M	RAEB	RAEB-1	10	Not evaluable		
24	75/M	RAEB	RAEB-2	15	Normal	2 INT2	6.5 VH

Abbreviations: adm, additional materials; AML, acute myeloid leukemia; ct, complex translocation; F, female; FAB, French–American–British classification; HIGH, high risk; INT2, intermediate 2; IPSS, International Prognostic Scoring System; M, male; RAEB, refractory anemia with excess blasts; RAEB-T, refractory anemia with excess blasts in transformation; r-IPSS, revised International Prognostic Scoring System; VH, very high; WHO, World Health Organization.

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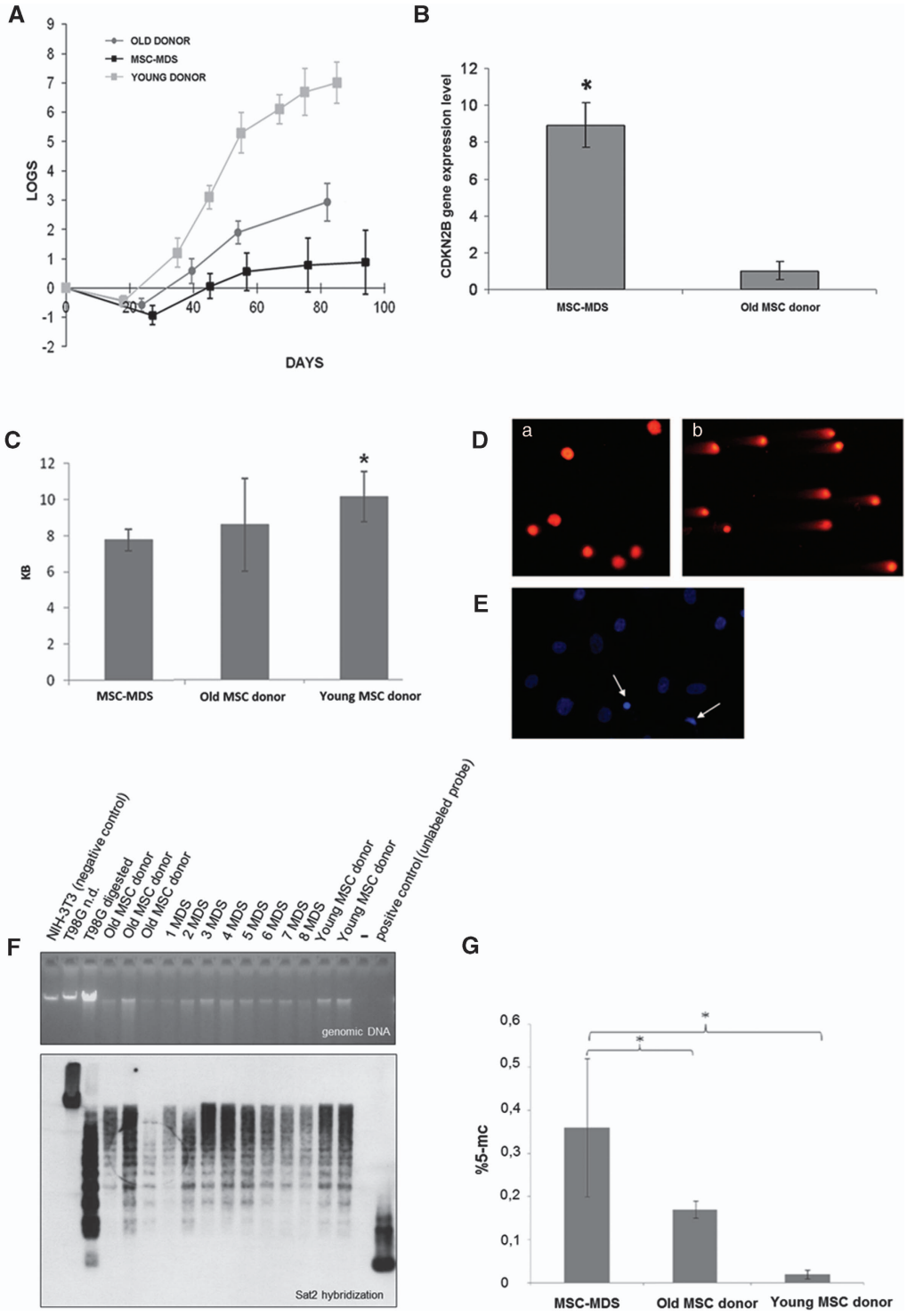

Figure 1. (**A**) Expansion capacity of the BM-derived MDS-MSCs calculated at every passage according to the equation: $\log_{10}$ (number of harvested cells/number of seeded cells). Finite logs were determined by the cumulative addition of the total numbers generated from each passage until the cells stopped dividing. The results are shown as the average values ± s.d. Each point on the growth curve represents a single passage of culture. (**B**) Molecular analysis for CDKN2B expression in MDS-MSC samples, in old and young MSC donors. The samples were run in duplicate and the values are the average ± s.d. *$P<0.05$. (**C**) Southern blot analysis of telomere length in the MDS-MSCs, in older and younger MSC donors. The values are the average ± s.d. *$P<0.05$. (**D**) Single cell gel electrophoresis assay. (a) No comet tails were seen in MDS-MSCs, suggesting that there was no damaged DNA (containing cleavage and strand breaks), magnification × 10. (b) DNA damage of treated cell line A549 was separated from intact DNA, yielding a comet tail shape, magnification × 10. (**E**) Hoechst 33342 staining in MDS-MSC samples for apoptotic cells, magnification × 10. (**F**) Southern blot analysis of satellite 2 methylation levels. (Upper panel) Csp45I-digested genomic DNA separated by 1% agarose gel electrophoresis, before blotting; (lower panel) satellite 2 hybridization of digested genomic DNA by using a PCR probe (sat2-384U/781L). Genomic DNA, digested with Csp45I, from T98G glioblastoma cell line has been used as control of satellite 2 demethylation. (**G**) Methylated DNA quantification assay of the MDS-MSCs, in older and younger MSC donors. The values are the average ± s.d. *$P<0.05$.

was in line with the clonogenic potential of MDS-MSCs performed with colony-forming unit fibroblast (CFU-F) assay, plating 5×10^6 mononuclear cells. CFU-F count of MDS samples was significantly reduced in comparison with healthy donors (clonogenic potential of MDS-MSCs vs old donors: 9 ± 6 vs 53 ± 26 CFU-F).

To study the ability to provide long-term hematopoietic support, peripheral blood progenitor CD133$^+$ cells isolated from leukapheresis of patients undergoing autologous transplantation were co-cultured onto feeder-layers of irradiated MDS-MSCs.[6] These cells were able to support the proliferation of both myeloid and erythroid progenitor cells but not to the level of MSCs from older donors (38 ± 15 vs 62 ± 20 colony-forming units, respectively) ($P < 0.05$).

The significantly reduced capacity of MDS-MSCs for proliferation together with the morphological changes suggested possible alteration in cell cycle progression and, as reported by other authors, a possible senescent-like phenotype.[7] Therefore, we studied the gene expression profiles for 37 genes (directly or indirectly involved in the cell cycle regulation) in MDS-MSCs at second culture passage.[8] The only significant difference found was represented by CDKN2B upregulation in the MDS-MSCs with an expression level of 8–11 times higher than that in the MSCs from older and younger donors ($P < 0.05$) (Figure 1B). These data may be associated with a negative regulation of the cell cycle. They may be at least partially responsible for the reduced proliferation potential and there could be a premature cellular senescence-like phenotype induction. Upregulation of CDKN2B has been indicated as being at least part of the molecular mechanisms responsible for the induction of senescence in MSCs. Recent data revealed that long-term culture induces continuous changes in gene expression; in particular an upregulation of CDKN2B in late-culture passages has been demonstrated, indicating an association between replicative senescence in long-term culture and this gene. Interestingly, in MDS-MSCs CDKN2B appeared overexpressed in early-culture passage demonstrating an impaired regulation of cell cycle progression independently from culture conditions.[9] However, we cannot detect any other elaboration of the gene expression that often relates to a senescence phenotype, such as the upregulation of CDKN1A and CDKN2A. In an attempt to elucidate the biological status of MDS-MSCs, we studied the presence of senescence-associated markers such as expression of beta-galactosidase, telomere shortening and genomic instability. Quantitative data of beta-galactosidase expression showed that there was no statistical difference between MDS-MSCs and healthy donors. We then evaluated the telomere length of MDS-MSC samples and controls using the telomere restriction fragment length assay.[10] The MDS-MSCs had shorter telomeres than the controls, but this difference was not statistically significant (7.8 ± 0.6 vs 8.6 ± 2.6 kb, respectively), although both MDS-MSCs and MSCs from older donors exhibited significantly shorter telomeres than MSCs from younger donors (7.8 ± 0.6 vs 10.1 ± 1.4 kb, respectively) ($P < 0.05$) (Figure 1C), as expected. In addition, MSC-MDS did not show signs of DNA damage once analyzed by comet assay (Figure 1D). Moreover, MDS-MSCs were stained with Hoechst 33342 to evaluate chromatin condensation and nuclear fragmentation, showing a low frequency of apoptotic cells (range 5–10%), similar to the MSCs of healthy donors (Figure 1E).

Globally reduced CpG methylation levels are often reported not only in cancer cells but also correlated with a premature senescent status. It is well known that replicative senescence of normal human fibroblast is accompanied by decreased methylation levels of heterochromatic sequences such as satellite 2 and 3.[11] Moreover, fibroblast-replicative senescence can be anticipated (premature senescence) by treatment with demethylating agents.

In function of its correlation with both replicative- and premature-senescence, and also as part of the phenomenon of global DNA hypomethylation in cancer, pericentromeric satellite 2 (Sat2) methylation was investigated in MDS-MSCs. Unexpectedly, the methylation-sensitive Sat2 digestion assay showed equal high levels of methylation between MSCs from donors and MDS-MSCs (Figure 1F). Thus, the global methylation levels of MDS-MSCs were further investigated comparing it with MSCs of older and younger healthy donors using 5-methyl-cytosine enzyme-linked immunosorbent assay quantification assay. In a recent work,[12] we demonstrated a global hypermethylated DNA status of hematopoietic cells in 134 MDS biopsies at diagnosis and this result correlated with the prognosis of these patients. Also, the MDS-MSCs exhibited globally hypermethylated DNA that was 2.2 times higher than that of the MSCs from older and younger healthy donors ($0.36 \pm 0.16\%$ vs $0.16 \pm 0.02\%$ and $0.02 \pm 0.01\%$, respectively) ($P < 0.05$) (Figure 1G).

The degree of aberrant methylation correlated with reduced mRNA and decreased protein expression and appeared to be associated with higher expression of DNMT3a (DNA methyltransferase 3a).[13] However, gene expression data obtained by real-time quantitative PCR approaches[11] from the MDS-MSCs showed no differences in the levels of DNMT1, DNMT3a and DNMT3b with respect to the donor MSCs.

Recently, Geyh *et al.*[7] conducted a detailed analysis of the molecular and functional properties of MDS-MSCs. They demonstrated a hypermethylation pattern of MDS-MSCs correlating it with impaired growth capacities and osteogenic differentiation. In particular, they found a hypermethylation status in the TBX15 and HOXB1 promoter region that resulted in reduced mRNA expression. They also showed the hypermethylation of a PTX2 gene body that resulted in a significant overexpression of PTX2 mRNA in MDS-MSCs. In line with these data, the reduced proliferative potential in the MDS-MSCs was directly linked with functional alterations. Neither increased apoptosis nor cell cycle defects were observed in MDS-MSCs, so their impaired proliferative capacity and altered morphology may be due to increased senescence. However, the absence of SA-β-Gal expression in MDS-MSCs in our data excluded some senescence mechanisms often involved in MDS-MSC-impaired growth, which is in line with a recently published observation.[14] The data demonstrated that premature cell senescence seems unlikely to account for the impaired proliferative potential of MDS-MSCs, which express fewer senescence characteristics such as the downregulation of CDKN1A, CDKN2A, CDKN2B and PARG1 compared with their normal counterparts. However, the low proliferation rate of MDS-MSCs might be associated with the upregulation of WNT signaling.

Moreover, other authors[15] analyzed MDS-MSC transcriptomes and demonstrated that these cells have an intrinsically altered pattern of gene expression, including a number of processes involved in intercellular crosstalk, that may contribute to their capacity to support MDS hematopoietic cells.

Our data showed that MDS-MSCs were structurally, epigenetically and functionally altered. The alterations indicated impaired stromal support and the possible involvement of MSCs in the ineffective MDS hematopoiesis. Moreover, the mechanisms underlying the impaired proliferative potential and cellular methylation status of MSCs are interesting avenues for further investigation. In conclusion, we suggest that MSCs could have a critical role in MDS pathogenesis and may be a possible target for new therapeutic strategies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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