

ORIGINAL ARTICLE

Mesenchymal stem cells from Shwachman–Diamond syndrome patients display normal functions and do not contribute to hematological defects

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Shwachman–Diamond syndrome (SDS) is a rare inherited disorder characterized by bone marrow (BM) dysfunction and exocrine pancreatic insufficiency. SDS patients have an increased risk for myelodysplastic syndrome and acute myeloid leukemia. Mesenchymal stem cells (MSCs) are the key component of the hematopoietic microenvironment and are relevant in inducing genetic mutations leading to leukemia. However, their role in SDS is still unexplored. We demonstrated that morphology, growth kinetics and expression of surface markers of MSCs from SDS patients (SDS-MSCs) were similar to normal MSCs. Moreover, SDS-MSCs were able to differentiate into mesengenic lineages and to inhibit the proliferation of mitogen-activated lymphocytes. We demonstrated in an *in vitro* coculture system that SDS-MSCs, significantly inhibited neutrophil apoptosis probably through interleukin-6 production. In a long-term coculture with CD34⁺-sorted cells, SDS-MSCs were able to sustain CD34⁺ cells survival and to preserve their stemness. Finally, SDS-MSCs had normal karyotype and did not show any chromosomal abnormality observed in the hematological components of the BM of SDS patients. Despite their pivotal role in the hematopoietic stem cell niche, our data suggest that MSC themselves do not seem to be responsible for the hematological defects typical of SDS patients.

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INTRODUCTION

Shwachman–Diamond syndrome (SDS) is a rare autosomal recessive inherited disorder characterized by bone marrow (BM) dysfunction and exocrine pancreatic insufficiency. In almost all affected patients, the disorder is characterized by a broad range of additional clinical features, including hematological alteration, with neutropenia as the most common deficiency, frequently associated with anemia, thrombocytopenia, defects in the lymphoid lineage, increased levels of fetal hemoglobin and aplasia. Development of the latter disorder suggests that the hematopoietic defect may arise at the level of an early hematopoietic stem cell (HSC) or in the BM stroma. In addition, similarly to other marrow failure syndromes, SDS patients show an increased risk of myelodysplastic syndrome (MDS) and malignant transformation, in particular to acute myeloid leukemia. Although SDS represents an important model for understanding genetic determinants in hematopoiesis and in developmental biology, the mechanisms underlying the BM dysfunction observed in SDS is not fully understood. In 1999, Dror and Freedman¹ reported a general marrow defects in SDS patients with abnormal BM stroma in terms of ability to produce fat clusters and to support and maintain hematopoiesis.

The key component of BM stroma are mesenchymal stem cells (MSCs), which represent the pivotal organizers for the generation,

maintenance and plasticity of the HSC niche by supporting the proliferation and differentiation of HSCs and their progenies. MSCs generate a number of stromal cells, which have been shown to have an impact on HSC behavior, including adipocytes,² pre-osteoblast,³ osteoblasts and chondrocytes.⁴ The hematopoietic microenvironment controls the formation of blood cells through the release of cytokines and extracellular matrix molecules.

Recently, Raaijmakers *et al.*⁵ demonstrated that targeted deletion of miRNA processing endonuclease *Dicer1* in osteoprogenitor cells in the BM could induce complex secondary changes in the organization of the hematopoietic lineages, including the development of independent genetic mutations and frank leukemia. This model, for the first time, candidates MSCs as new actors in the multi-hit process of oncogenesis, as an expression of the involvement of hematopoietic microenvironment in the pathophysiology of the disease. Several studies demonstrated the presence of chromosome alterations in MSCs derived from MDS or leukemic patients,^{6,7} suggesting that unstable MSCs may facilitate the expansion of malignant cells. Whether MSC alterations influence hematological disorders and how such alterations contribute to the progression of the disease remains controversial.

In this study, for the first time, we deeply characterized MSCs from SDS patients (SDS-MSCs) by investigating their possible

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defects in supporting hematopoiesis and their potential role in promoting malignant transformation.

MATERIAL AND METHODS

Patients

BM samples, derived from 27 out of 80 SDS patients enrolled in the 'SDS' Italian registry, were collected from exceeding diagnostic specimens. Table 1 shows the main clinical and genetic characteristics of all SDS patients enrolled in this study. All patients were diagnosed as SDS based on clinical criteria, including clear evidence of both hematological and exocrine pancreatic dysfunction and were positive for the *SBDS* gene mutation. The median age at enrollment was 9 years (range 1–27). All patients did not show any tumor evidence at the time of MSC generation and at 1 year of follow-up. The severity of neutropenia was determined according to absolute neutrophil count (ANC) (ANC <1500 = mild neutropenia, ANC <500 = severe neutropenia, ANC <200 = very severe neutropenia). BM samples from 25 healthy donors (HDs) were obtained from collection bags, discarded after BM infusion. Informed, written consent approved by local ethical committee was obtained in all cases.

Isolation and culture of MSCs

Mononuclear cells were isolated from BM using Ficoll-Paque PLUS (GE Healthcare, Waukesha, WI, USA) and seeded at 16×10^4 cells per cm^2 in Dulbecco's Modified Eagle Medium low glucose (Lonza, Basel, Switzerland) supplemented with 10% of fetal calf serum (Biosera, Ringmer, UK) at 37 °C with 5% CO_2 . After 24 h, nonadherent cells were removed. Adherent cells were trypsinized after reaching 70–80% confluence, and seeded at 0.3×10^4 cells per cm^2 . MSCs were used for the experiments between passage (P)3 and P5.

To study MSC growth pattern, adherent cells were trypsinized and counted by 0.4% trypan blue exclusion method (dead cells were stained by trypan blue and thus excluded) at different time points. The number of population doubling was calculated from P2, when the cell population is

homogeneous in terms of morphology and phenotype, up to P5, with the following formula: population doubling = $t \log 2 / (\log N_t - \log N_0)$.⁸

Phenotypic characterization of SDS-MSCs

To characterize expanded MSCs, the following monoclonal antibodies were used, according to the manufacturer's instructions: allophycocyanin-labeled anti-CD11b (Biolegend, San Diego, CA, USA); phycoerythrin (PE)-labeled anti-CD14 (eBioscience, San Diego, CA, USA); peridinin chlorophyll protein-labeled anti-CD19 (Becton Dickinson (BD), Franklin Lakes, NJ, USA); fluorescein isothiocyanate-labeled anti-CD34 (IQ products, Groningen, The Netherlands); PE-labeled anti-CD45 (BD); PE-labeled anti-CD73 (BD); PE-labeled anti-CD90 (eBioscience); PE-labeled anti-CD105 (eBioscience); fluorescein isothiocyanate-labeled anti-HLA-ABC (BD) and PE-labeled anti-HLA-DR (BD). Samples were acquired by FACScalibur (BD) and analyzed by CellQuest Software (Becton Dickinson, Inc., Oakville, ON, Canada).

Multilineage differentiation

Adipogenic and osteogenic differentiating ability of MSCs was determined at P5 as previously described.⁹ Briefly, osteogenic differentiation was evaluated by Alizarin Red (Sigma-Aldrich, St Louis, MO, USA) staining and adipogenic differentiation by Oil Red O (Sigma-Aldrich) staining. Quantitative reverse transcriptase PCR was used to determine the expression of the following genes involved in the differentiation process: leptin (*LEP*), lipoprotein lipase (*LPL*), peroxisome proliferator-activated receptor gamma (*PPAR-gamma*), alkaline phosphatase (*ALKP*), osteocalcin (*OCN*) and osteopontin (*OPN*). Glyceroldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as housekeeping gene. All quantitative reverse transcriptase PCR analysis were performed on LightCycler 480 (Roche, Basel, Switzerland) using Universal Probe Library probes. Samples were run in triplicate. Gene expression relative to *GAPDH* was quantified by the $2^{-\Delta\Delta C_t}$ method¹⁰ and indicated as fold change. Chondrogenic differentiation was determined as previously described.⁹ Accumulation of cartilage matrix, proteoglycans and glycosaminoglycans was evaluated by hematoxylin-eosin and safranin/fast green staining.

Table 1. Clinical and genetic characteristic of the 27 patients enrolled in the study

Patients	Sex	Age at study (years)	ANC/ μl	Infections	<i>SBDS</i> mutations	Karyotype
UPN1	F	7	1100	NO	c.258 + 2T > C	46,XX
UPN2	F	3	1770	NO	c.258 + 2T > C	46,XX[20]
UPN3	F	4	1820	NO	c.258 + 2T > C	46,XX,i(7)(q10)[28]/46,XX[1]
UPN4	F	3	1440	NO	c.258 + 2T > C	46,XX[7]
UPN5	F	1	260	NO	c.258 + 2T > C	46,XY[23]
UPN6	F	8	100	NO	c.258 + 2T > C	46,XX,i(7)(q10)[5]/46,XX[16]
UPN7	M	6	1600	URTI	c.258 + 2T > C	46,XY[15]
UPN8	F	1	260	NO	c.258 + 2T > C	46,XX[17]
UPN9	M	18	380	NO	c.258 + 2T > C	46,XY[20]
UPN10	F	4	1100	NO	c.258 + 2T > C	46,XY[9]
UPN11	M	4	2400	NO	c.258 + 2T > C	ND
UPN12	M	19	950	NO	c.258 + 2T > C	46,XY[13]
UPN13	M	27	1120	NO	c.258 + 2T > C	46,XY[20]
UPN14	F	9	1000	NO	c.258 + 2T > C	46,XX[23]
UPN15	M	20	250	NO	c.258 + 2T > C	46,XY[24]
UPN16	M	10	2350	NO	c.258 + 2T > C	ND
UPN17	F	6	1620	NO	c.258 + 2T > C	46,XX[24]
UPN18	F	7	1760	ND	c.258 + 2T > C	46,XX[22]
UPN19	M	3	2960	NO	c.258 + 2T > C	46,XY[26]
UPN20	F	15	278	NO	c.258 + 2T > C	ND
UPN21	M	18	1500	NO	c.258 + 2T > C	46,XY,i(7)(q10)[1]/46,XY[6]
UPN22	M	11	310	ND	c.258 + 2T > C	46,XY,i(7)(q10)[4]/46,XY[16]
UPN23	M	13	800	ND	c.258 + 2T > C	46,XY,del(20)(q11,21q13,32)[6]/46,XY [7](q10)[1]/46,XY[6]
UPN24	F	12	ND	NO	c.258 + 2T > C	46,XX[10]
UPN25	M	3	820	ND	c.258 + 2T > C	46,XY[9]
UPN26	F	7	ND	NO	c.258 + 2T > C	46,XX[31]
UPN27	M	7	ND	ND	c.258 + 2T > C	46,XY[17]

Abbreviations: ANC, absolute neutrophil count; F, female; M, male; ND, not detected; PB, peripheral blood; UPN, unique patient number; URTI, upper respiratory tract infection. All mutations are described according to the mutation nomenclature (www.hgvs.org/mutnomen).

Proliferation assay

Peripheral blood mononuclear cells were stimulated with 5 µg/ml of phytohemagglutinin (PHA) (Irvine Scientific, Santa Ana, CA, USA) and cocultured with different doses of irradiated (35 Gray) MSCs in 96-well plates. After 48 h of coculture cells were pulsed for 16 h with [3H]-thymidine at 1 µCi per well (PerkinElmer, Waltham, MA, USA) and then harvested. [3H]-thymidine incorporation was measured using Multipurpose Scintillation Counter (Beckman Coulter, Brea, CA, USA).

Neutrophil and MSC coculture

In order to obtain a pure and minimally activated cell population, neutrophils were isolated from peripheral blood of HDs under endotoxin-free conditions following the protocol described by Boyum.¹¹ After purification, the mean neutrophil purity was 95%, as determined by morphological and phenotypic analyses. Neutrophils were then layered on MSC or cultured alone. At 24 and 48 h, cultured neutrophils were recovered, counted and analyzed for viability by Apoptosis/Necrosis detection kit (Enzo Life Sciences, Farmingdale, NY, USA) and flow-cytometry analysis. Supernatants from the coculture were used to evaluate the amount of interleukin-6 (IL-6) by enzyme-linked immunosorbent assay (eBioscience), according to the manufacturer's instruction.

HSC and MSC coculture

CD34⁺ cells were isolated from BM of HD by anti-CD34 antibody-conjugated magnetic beads, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). CD34⁺ cells were suspended in complete Roswell Park Memorial Institute medium and plated at a density of 5×10^4 cells per cm² on a confluent irradiated (35 Gray) MSC layer in 48-well plate. Phenotype and short-time colony assay of CD34⁺ cells were assessed weekly. A total of 10^3 cells were resuspended in Iscoves Modified Dulbecco's Media (Euroclone, Pero, Italy) and plated in 10% methylcellulose-based medium (STEMCELL Technologies, Vancouver, BC, Canada) supplemented with 1% of pen-strep, L-glutamine, 2U of erythropoietin (Roche) and 10% 5637 conditioned medium. The conditioned medium from 5637 tumor cell line was used as a source of colony-stimulating factor at a final concentration of 10%. After 21 days of incubation at 37 °C in a fully humidified atmosphere of 5% CO₂, colony-forming unit were scored under an inverted microscope. Aggregates of > 50 cells were evaluated as colonies.

Western blot analysis

MSCs (1.5×10^6) were collected at P5 and were lysed in radioimmunoprecipitation assay buffer as already described.¹² A total of 50 µg of protein was loaded per lane on a 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane (Life Technologies, Paisley, UK). The following antibodies were used: goat polyclonal anti-SBDS (1/800) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-β-actin (loading control, 1/20000) (Sigma-Aldrich) in PBST/5% of milk.

Karyotype and fluorescence in situ hybridization (FISH) analysis of SDS-MSCs

MSCs were cultured in medium supplemented with 10 µl/ml of colcemid (10 g/ml, Sigma) for up to 6 h. Conventional cytogenetics was performed by the standard procedures and QFQ (Q-bands by fluorescence using quinacrine) banding. The karyotypes were described according to the International System for Human Cytogenetic Nomenclature (2009).¹³ FISH analysis was performed on MSCs using LSI D20S108 probe (Vysis, Downers Grove, IL, USA) and D7S486/CEP7 FISH Probe Kit (Vysis) and on BM patients using D7S486/CEP7 FISH Probe Kit (Vysis) and BAC RP11-17F3 (20q12). The FISH cut-off for each probe was determined analyzing MSCs derived from HDs. The probes cut-off level was 6% for LSI D20S108 and 2% for D7S486/CEP7. At least 200 nuclei were analyzed for each case using Isis software (v.5.4.9; MetaSystems, Altlußheim, Germany) and Imager Z1 (Zeiss, Oberkochen, Germany) epifluorescence microscope equipped with 4',6-diamidino-2-phenylindole, fluorescein isothiocyanate and TEXAS RED filters.

Statistical analysis

All values are expressed as mean ± s.d. or s.e. To evaluate statistical significance between the groups, Student's *t*-test was used. A *P*-value < 0.05 was considered to be statistically significant.

RESULTS

Evaluation of MSC-typical features in cells expanded from BM of SDS patients

MSCs were obtained from BM of 27 SDS patients and were characterized for their proliferating ability, antigen expression and differentiation into mesengenic lineages. SDS-MSCs displayed a typical fibroblast-like morphology, similarly to HD-MSCs (data not shown). Flow-cytometric analysis showed that SDS-MSCs, as well as HD-MSCs, were negative for hematopoietic (for example, CD34, CD45 and MHC class II), lymphoid (for example, CD19) and myeloid (for example, CD11b and MHC class II) markers and positive for stromal cell-associated markers (for example, CD73, CD90 and CD105) (Figure 1a). Adipocytic, osteoblastic and chondrocytic differentiation assays were performed for SDS-MSCs. SDS-MSCs were able to differentiate into osteoblasts, as demonstrated by the histological detection of calcium deposition by Alizarin Red staining (Figure 1b, on the left). Osteogenic differentiation was also confirmed by real-time PCR, under osteogenic permissive condition at day 14 of culture. Expression of *ALKP*, *OCN* and *OPN* osteogenic genes resulted similar in SDS-MSCs and HD-MSCs (Supplementary Figure 1A). In addition, SDS-MSCs were able to differentiate into adipocytes, as revealed by the formation of lipid droplets visualized by Oil Red O staining (Figure 1b, in the middle). Similarly to HD-MSCs, SDS-MSCs increased the expression of *PPAR-γ*, *LEP* and *LPL* during the adipogenic differentiation process (Supplementary Figure 1B). Concerning the chondrogenic differentiation, cultured pellets became free-floating within the first 24 h of induction. During the differentiation process, biosynthesis and deposition of extracellular matrix components, such as sulfated proteoglycans, contributed to increase cell pellet size, as demonstrated by hematoxylin-eosin (Figure 1b, on the right), safranin and fast green staining (inner panel).

To evaluate their proliferating ability, SDS-MSCs were subcultured when they reached 70–80% of confluence and population doubling time was calculated at each passage point from P2 to P5. Proliferation rate was comparable at each time point considered (Student's *t*-test > 0.05) between SDS- and HD-MSCs (Figure 1c). Interestingly, cell passaging reduced the proliferation rate in all MSCs tested. In particular, 4 days (± 1.2 for SDS-MSCs and ± 1.4 for HD-MSCs) were necessary to double cell number of both SDS-MSCs and HD-MSCs at P2, whereas population doubling was 4.3 ± 1.4 days for SDS-MSCs and 3.7 ± 0.9 days for HD-MSCs at P5.

We then evaluated the immunoregulatory activity of SDS-MSCs by assessing their ability to inhibit lymphocyte proliferation induced by mitogenic stimuli (that is, PHA). Peripheral blood mononuclear cells from HD were stimulated with PHA in the presence or absence of various concentrations of irradiated MSCs. SDS-MSCs were able to strongly suppress lymphocyte proliferation in a dose-dependent manner, with a 58% of inhibition observed at 1:32 SDS-MSC/peripheral blood mononuclear cell ratio (range, 24–99%), similarly to HD-MSCs (Figure 1d).

Functional characterization of SDS-MSCs

With the aim to evaluate the functional properties of SDS-MSCs, we analyzed their capability to preserve neutrophils from apoptosis and to maintain the viability of CD34⁺ cells. Neutrophils are very sensitive cells and once released from the BM they circulate in the peripheral blood with a short half-life. We investigated the effects of MSCs on spontaneous neutrophil apoptosis, by culturing HD neutrophils in the absence or presence of SDS-MSCs. Because Raffaghello *et al.*¹⁴ demonstrated that inhibition of neutrophil apoptosis occurred at a MSC:neutrophil ratio ranging from 1:1 to 1:500, we used a MSC:neutrophil ratio lower than 1:50 for all the coculture experiments. As shown in Figure 2a, after 24 h only 3% of neutrophils cultured with medium

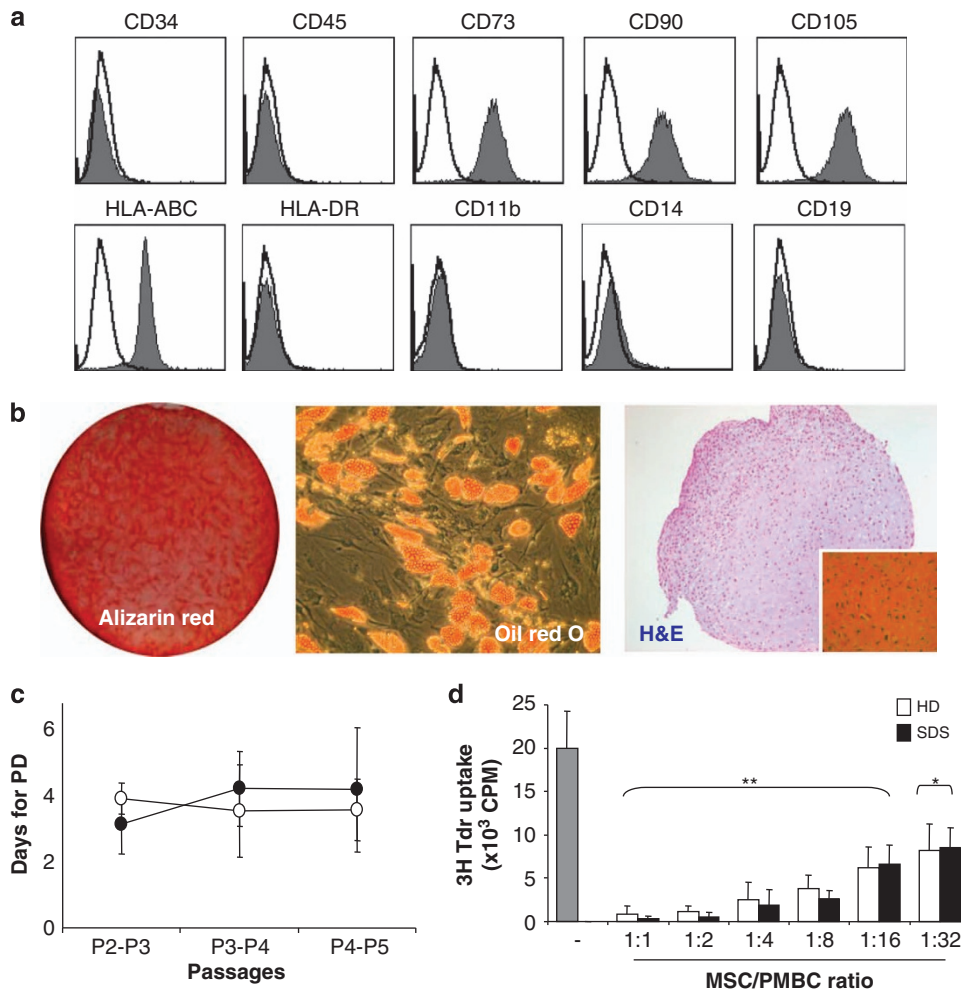


Figure 1. Isolation and expansion of SDS-MSCs. MSCs were isolated from bone marrow of SDS patients and cultured in Dulbecco's Modified Eagle Medium with 10% of fetal calf serum. **(a)** Fluorescence-activated cell sorting (FACS) analysis of SDS-MSCs. Isotype control staining (white area under continuous line) versus the specific antibody staining profile (gray area) is shown. Histograms from 1 representative experiment out of 27 performed are shown. **(b)** Mesengenic differentiation of SDS-MSCs. To detect the osteogenic differentiation, cells were stained for calcium deposition with Alizarin Red (on the left) and adipogenic differentiation was evaluated through the morphological appearance of fat droplets with Oil Red O (in the middle). Cell pellet was treated with chondrogenic medium for 21 days, sections of cell pellet were stained with hematoxylin-eosin (on the right) and Safranin O (inner panel), which binds proteoglycans and glycosaminoglycans. Canon PowerShot G5, magnification $\times 10$. One experiment out of twenty-seven performed is shown. **(c)** Expansion curve of SDS-MSCs. The graph represents the mean \pm s.e. of 25 HD-SDS versus 27 SDS-MSC. Population doubling (PD) = $t \log 2 / (\log N_t - \log N_0)$, where t represents culture time, N_0 and N_t represent cell number before and after seeding, respectively. **(d)** Lymphocyte proliferation in the presence or absence of SDS-MSCs. Peripheral blood mononuclear cells (PBMCs) were stimulated with PHA in the absence (–) or presence of different concentration of MSCs (–) in 96-well plates. After 48 h, cells were pulsed for 16 h with [3 H]-thymidine at $1 \mu\text{Ci}$ per well (PerkinElmer) and then harvested. The graph represents the mean \pm s.e. of eight experiments (** $P < 0.01$ and * $P < 0.05$, versus PBMC stimulated with PHA in absence of MSC).

alone resulted viable. This percentage further decreased after 48 h of culture to 1% of viable cells. In contrast, HD-MSCs and SDS-MSCs were comparably able to support the viability of neutrophils at both 24 and 48 h (Figure 2a). In particular, at 24 h, 7.6% (range 3–16%) and 13% (range 4–23%) of neutrophils were alive (double negative cells) when cocultured with HD-MSCs or SDS-MSCs, respectively. It has been demonstrated that IL-6 is constitutively produced by MSCs and has a crucial role in inhibiting neutrophil-programmed cell death. For this reason, we evaluated IL-6 concentration in supernatants recovered from SDS-MSCs and neutrophil coculture. SDS-MSCs were able to produce IL-6 in a time-dependent manner (Figure 2b). In particular, IL-6 production increased after 72 h of culture with both SDS- and HD-MSCs, with no statistically significant differences between the two groups.

In order to assess the ability of SDS-MSCs to support and maintain hematopoiesis, we took advantage of long-term cultures to mimic the interactions between the hematopoietic progenitors and the microenvironment of the BM stem cell niche *in vitro*. With this aim, CD34⁺-sorted cells were cocultured with MSCs from both patients and controls. Total number of nonadherent cells was calculated during the first 3 weeks of coculture and was found to be similar. After an initial decline at day 7, the total number increased after 14 days of coculture (Figure 3a). As expected, after 21 days, the percentage of CD34⁺ cells diminished up to 20% (Figure 3b). Importantly, SDS-MSCs as well as HD-MSCs were able to preserve the stemness of recovered cells, as evaluated by their ability to form CFU-GM (colony forming unit-granulocyte/macrophage) colonies in methylcellulose. No statistical differences in the number of CFU-GM

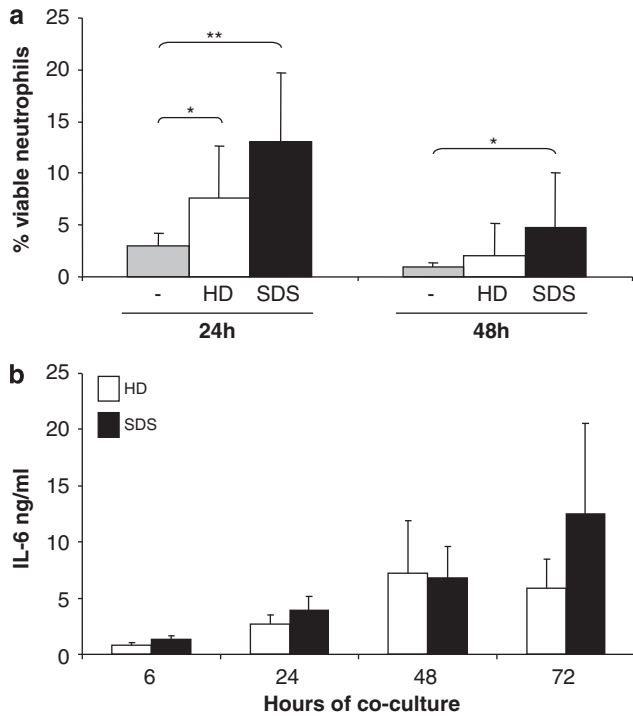


Figure 2. (a) Evaluation of neutrophil viability cultured with SDS-MSCs. Neutrophils from HDs were cultured alone or in the presence of HD-MSCs and SDS-MSCs at 24 and 48 h. Viable neutrophils were evaluated by annexin V and 7-AAD (7-aminoactinomycin D) staining as percentage of double negative cells on recovered cells at each time point analyzed. The mean percentage of viable cells evaluated in 10 independent experiments is shown. (b) IL-6 secretion after neutrophils and SDS-MSCs coculture. Quantification of IL-6 in the supernatants was detected by enzyme-linked immunosorbent assay at different time points. The graph represents the mean \pm s.e. of 10 experiments (Student's *t*-test >0.05).

colonies (Figure 3c) obtained from CD34⁺ cells cocultured with both SDS-MSCs and HD-MSCs were observed.

Analysis of SBDS protein in SDS-MSCs

It has been demonstrated that SBDS protein shows low expression levels in all hematopoietic cell lineages from SDS patients.¹⁵ In order to assess the level of SBDS protein in SDS-MSCs, we performed western blot analysis on lysates from seven SDS-MSCs and eight HD-MSCs. In line with previous studies on BM,¹⁵ SBDS protein was undetectable in all SDS-MSCs (lanes 1–3, Figure 4). On the contrary, a variable amount of protein was detected in HD-MSC (lanes 4–8).

Karyotype and FISH analysis of SDS-MSCs

To study the potential role of MSCs in the predisposition to leukemia or MDS of SDS patients, we focused on the cytogenetic characterization of SDS-MSCs. With this aim, we performed conventional cytogenetic and FISH analysis to explore genomic alterations in MSCs obtained from 4 out of 5 patients carrying chromosomal abnormalities, and to compare these with the aberrations observed in the BM of the same patients.

Karyotype and FISH analysis were performed in SDS-MSCs obtained from UPN3, UPN6, UPN21 and UPN23, in the BM sample and in MSCs from five healthy volunteers. The median number of MSC passages was 5. The culture time ranged from 29 to 35 days.

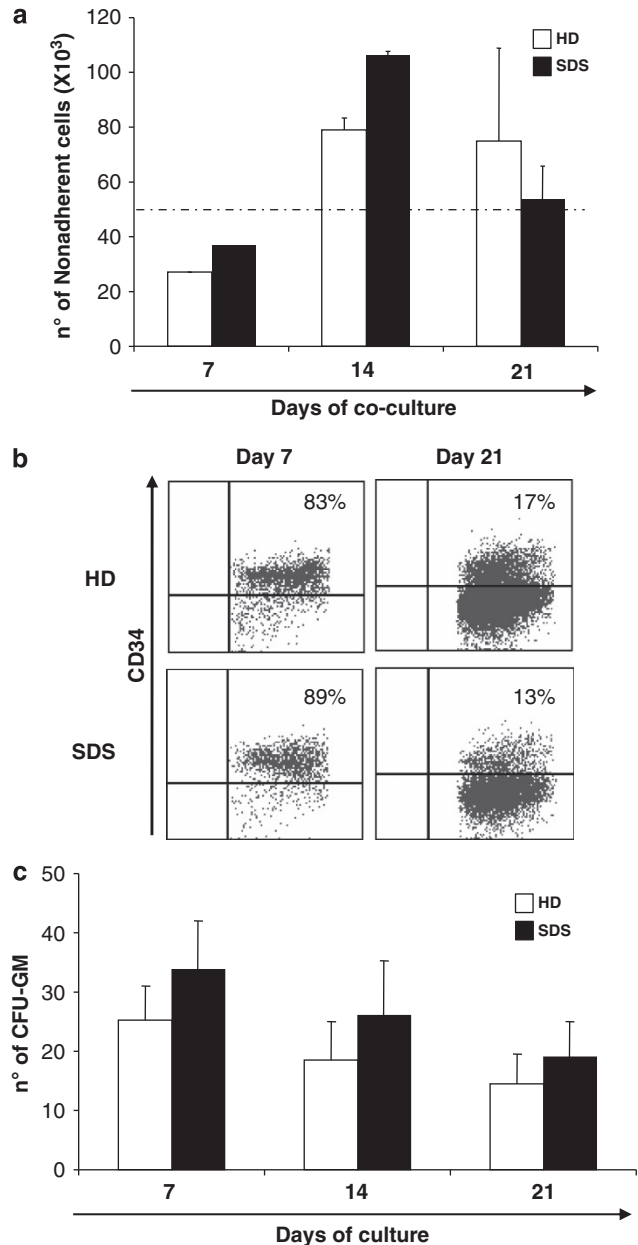


Figure 3. (a) Nonadherent cell numbers from long-term culture with SDS-MSCs. In all, 5×10^4 CD34⁺ cells from HDs were plated in wells containing irradiated MSCs (dotted line). Cells were harvested from individual wells after 7, 14 and 21 days. Data represent the mean of eight experiments performed \pm s.e., Student's *t*-test >0.05 . (b) FACS analysis of nonadherent cells obtained after long-term culture. Percentage of CD34⁺ cells from HD-MSCs or SDS-MSCs cocultures was shown. The experiment is representative of eight performed. (c) Number of CFU-GM derived from long-term culture nonadherent cells, plated weekly in short-term clonogenic assays. After 7, 14 and 21 days of coculture, 1000 of nonadherent cells were seeded in wells containing methylcellulose. Colonies were scored as CFU-GM using an inverted microscope after 21 days. Data represent the mean of eight experiments performed \pm s.e. (Student's *t*-test >0.05).

FISH analysis demonstrated that in none of the patients, SDS-MSCs displayed the chromosomal abnormality ($n = 0/200$ nuclei analyzed) observed in the respective BM sample (UPN3: i(7)(q10), $n = 124/200$; UPN6: i(7)(q10), $n = 49/549$; UPN21: i(7)(q10), $n = 30/300$ and UPN23: del(20)(q11), $n = 162/601$). Figure 5a shows the FISH analysis of a representative experiment.

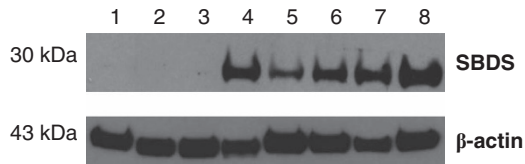


Figure 4. Detection of SBDS protein in SDS-MSCs by western blot. Proteins extracted from MSCs were analyzed by immunoblotting with the SBDS antiserum. β -actin protein level served as a loading control. SBDS protein is absent in SDS-MSCs. Shwachman patients are lanes 1, 2 and 3 (UPN16, UPN1 and UPN11, respectively); controls are lanes 4, 5, 6, 7 and 8. Molecular size markers are indicated on the left in kilodaltons.

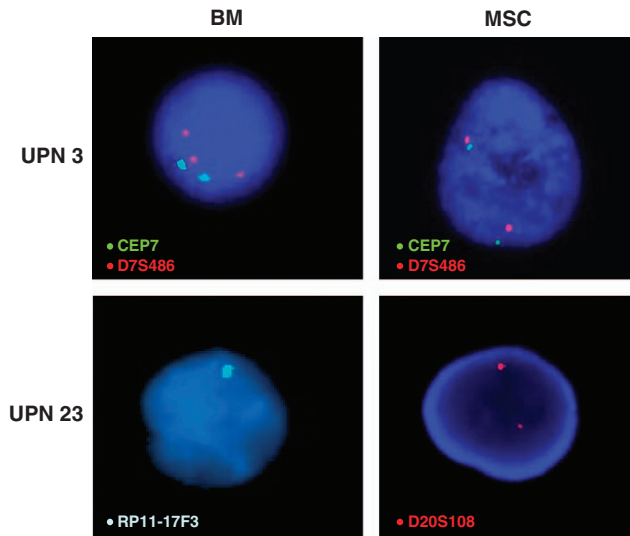


Figure 5. (a) FISH analysis performed on SDS-MSCs. The LSI D7S486/CEP7 (Vysis) and BAC RP11-17F3 probes were used to search for isochromosome 7q and 20q arm deletion in BM samples: UPN3 showed hybridization pattern consistent with the presence of isochromosome 7q (two green signals and three red signals); UPN23 revealed deletion of 20q (one aqua signal). SDS-MSCs derived from patients were tested for the same abnormalities using LSI D7S486/CEP7 (Vysis) and LSI D20S108 (Vysis) probes, and they did not reveal any aberrations.

Cytogenetic analysis of SDS-MSCs (20 metaphases in all cases) did not show any of the chromosomal alterations observed in the BM (UPN3: $i(7)(q10)$, $n = 28/29$; UPN6: $i(7)(q10)$, $n = 5/21$; UPN21: $i(7)(q10)$, $n = 1/7$; and UPN23: $del(20)(q11)$, $n = 6/13$) (Table 1). No other abnormalities, aneuploidy or polyploidy were observed in SDS-MSCs. Chromosome analysis of HD-MSCs showed normal diploid karyotype, with no aneuploidy, polyploidy or structural abnormalities (data not shown).

DISCUSSION

SDS is an autosomal recessive disorder characterized by hematological defects, exocrine pancreatic insufficiency and skeletal deformities. Patients may display a wide range of hematological abnormalities, but the number of neutrophils in peripheral blood samples is reduced (neutropenia) in all individuals affected. Using long-term cultures of marrow stromal cells from SDS and unaffected subjects, Dror and Freedman¹ demonstrated that patients show dysfunction of the BM stroma. In particular, a reduced ability to support and maintain hematopoiesis and a stem cell defect were observed. Until now, the role of MSCs in the

pathophysiology of the inefficient hematopoiesis observed in SDS is still unclear.

In the present study, we isolated and characterized MSCs from the BM of 27 patients. We demonstrated that SDS-MSCs are similar to HD-MSCs in terms of morphology and growth kinetics. Furthermore, flow-cytometry data demonstrated the expression of several cell-surface antigens, such as CD73, CD90 and CD105, commonly found on HD-MSCs. Moreover, SDS-MSCs were able to differentiate into adipocytes, osteoblasts and chondrocytes, under appropriate induction conditions. Finally, we demonstrated that SDS-MSCs strongly inhibit the proliferation of PHA-activated lymphocytes. Overall, these results demonstrated that cells with typical MSC features can be successfully obtained from the BM of SDS patients.

After characterizing SDS-MSCs, we tried to better understand their potential role in the hematopoietic insufficiency observed in SDS patients. Stromal impairment in SDS can be related to either the reduced expression of hematopoietic cytokines, the altered production of extracellular matrix or the abnormal expression of adhesion molecules necessary for the interaction between hematopoietic progenitors and stromal cells. We cocultured SDS-MSCs with $CD34^+$ cells and, in our culture condition, we observed that SDS-MSCs were able to support the viability and the stemness potential of $CD34^+$ cells as well as HD-MSCs. The altered function of SDS stroma reported by Dror¹ was demonstrated by *in vitro* experiments using BM stroma *in toto* as an integral unit of various cell types. In our experiments, we used isolated MSCs as precursors of a number of stromal cells, which have been shown to have an impact on HSC behavior. Despite their essential role in organizing and maintaining the HSC niche, our data suggest that MSCs themselves do not seem to be responsible for the BM failure typical of SDS.

We also analyzed the effect of SDS-MSCs on neutrophils and we demonstrated that, after coculture, neutrophils were protected from apoptosis. As demonstrated by Raffaghello *et al.*¹³ for normal MSCs, SDS-MSCs produced IL-6, a cytokine involved in prevention of excessive or inappropriate neutrophil activation. Overall, these results indicate that SDS-MSCs act *in vitro* just like their normal counterparts. Further *in vivo* studies mirroring the whole architecture of the HSC niche are needed to comprehend the possible MSC defects in SDS patients.

Patients analyzed in our study were diagnosed with SDS based on clinical criteria and the diagnosis was supplemented by positive *SBDS* gene mutation tests. In agreement with previous reports, the $258 + 2T > C$ and $183-184 > CT$ mutations were the most common in our cohort. Woloszynek *et al.*¹⁶ detected the SBDS protein in samples from family members with normal *SBDS* alleles but not in patients. Full-length SBDS protein was not detected in leukocytes of SDS patients with the most common *SBDS* mutations, consistent with a loss-of-function mechanism. We demonstrated that, even though *SBDS* gene transcript expression did not differ significantly between patients and controls (data not shown), SBDS protein was not detectable in SDS-MSCs. Recent studies in yeast and patient BM cells show that *SBDS* gene is involved in RNA metabolism and ribosome biogenesis.^{17,18} *SBDS* is an essential gene in embryogenesis and it is also implicated in cell division and cellular stress response.^{19,20} Further studies are needed to better comprehend the mechanisms compensating the absence of SBDS protein.

Similar to other marrow failure syndromes, SDS patients are cancer prone and have a risk of developing myelodysplastic syndrome and/or leukemia. Raaijmakers *et al.*,⁵ using transgenic mice, showed that genetic alteration of cells belonging to the BM microenvironment can induce MDS with ineffective hematopoiesis and with occasional transformation to acute myeloid leukemia. Although it is generally agreed that the marrow microenvironment has a role in the biology of hematological diseases, the mechanisms by which the stromal

compartment promote the malignant transformation is unclear. Different groups^{6,7,21–24} have shown the extensive variability of the aberrations, such as balanced and unbalanced translocations, hypodiploidy, deletions and whole-chromosome gains in MSCs obtained from leukemic patients. In view of these data, genetic alterations in MSCs may indicate a common origin and a potential interaction of MSCs in leukemia development and/or progression. To study the potential implication of MSCs in predisposing or promoting leukemia or MDS evolution in SDS patients, we analyzed their genetic features. In spite of having chromosomal abnormalities in the BM counterpart, we did not find any chromosomal alteration in SDS-MSCs. Probably, the most common chromosomal alterations [i(7)(q10) or del(20)(q11)] observed in SDS patients occurred in a more mature, already committed, progenitor instead of involving the mesenchymal compartment. The fact that SDS-MSC did not carry the same anomaly observed in the BM of patients does not exclude the presence of other genetic alteration; however, whole-genome single-nucleotide polymorphism array did not show any copy number aberration in MSC from the SDS patient tested. These results suggest that SDS-MSCs patients may be nonmalignant cells.

All SDS patients did not show any tumor evidence at the time of MSC generation and in 1 year follow-up. It would be of interest to continuously follow the patients and, in case of tumor evolution, evaluate the MSC genetic status at the time and early before the progression.

We described, for the first time at our knowledge, the isolation, expansion and functional characterization of SDS-MSCs. Our results showed that SDS-MSCs were similar to normal BM-derived MSCs in terms of morphology, growth property, surface epitopes and differentiation ability. Moreover, SDS-MSCs had normal karyotype and may be nonmalignant. Despite their pivotal role in the BM niche, our data suggest that MSC themselves do not seem to be responsible for the hematological defects typical of SDS patients. Our data give a new insight into biology of SDS and might have a positive impact of the management of HSC transplantation in SDS patients, by sustaining HSC transplantation despite the supposed risk of failure due to stroma abnormalities.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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