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ORIGINAL ARTICLE

Heterogeneity of Mesenchymal Stromal Cells in Myelodysplastic Syndrome-with Multilineage Dysplasia (MDS-MLD)

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Abstract Bone marrow niche constituents have been implicated in the genesis of clonal hematopoietic dysfunction in myelodysplastic syndromes (MDS), though the exact role of stroma in the pathogenesis of MDS remains to be defined. We have evaluated the characteristics of mesenchymal stromal cells in a cohort of patients with MDS with multilineage dysplasia (MDS-MLD). MSCs were cultured from bone marrow aspirates of MDS-MLD patients and controls with healthy bone marrow. Phenotypic characterization, cell cycle, and apoptosis were analyzed by flow cytometry. Targeted gene expression analysis was done using a reverse-transcription polymerase chain reaction (Q-PCR). MSCs derived from MDS patients (MDS-MSCs) showed normal morphology, phenotype, karyotype and differentiation potential towards adipogenic and osteogenic lineages. However, these MDS-MSCs showed significantly altered cell cycle status and displayed a shift towards increased apoptosis compared to control MSCs (C-MSCs). The gene expression profile of niche responsive/regulatory cytokines showed a trend towards lower expression *VEGF*, *SCF*, and *ANGPT* with no changes in expression of *CXCL12A* and *LIF* compared to C-MSCs. The expression levels of Notch signaling components like Notch ligands (*JAGGED-1* and *DELTA-LIKE-1*), receptors (*NOTCH1*, *NOTCH3*) and downstream gene (*HES1*) showed an aberrant expression pattern in MDS-MSCs compared to C-MSCs. Similarly, Q-PCR analysis of Wnt signaling inhibitory ligands (*DKK-1 and DKK-2*) in MDS-MSCs showed a three-fold increase in mRNA expression of *DKK1* and a two-fold increase in *DKK2* compared to C-MSCs. These data suggested that MDS-MSCs have an altered proliferation characteristic as well as a dysregulated cytokine secretion and signaling profile. These changes could contribute to the pathogenesis of MDS.

Keywords Myelodysplastic syndrome · Mesenchymal stromal cells · Hematopoietic stem · Progenitor cells

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Introduction

Mesenchymal stromal cells, characterized by their plastic adherence properties in culture and expression of CD73, CD90, CD105 antigens, without hematopoietic markers such as CD34, CD45 and CD14 [1], have been shown to be a key component of the bone marrow niche. They play a role in homing, self-renewal, differentiation and proliferation of hematopoietic stem cells (HSC) [2–4]. Furthermore, studies using genetically engineered mouse models have shown that different subsets of immature mesenchymal progenitor-like CXCL12 Abundant Reticular (CAR) Cells, nestin-positive MSCs, leptin-receptor (LepR) expressing MSCs have a regulatory role in controlling HSPC

functions, thus forming an essential component of hematopoietic niche [5–7].

MSCs isolated from MDS patients have been evaluated by in vitro assays or assessed in vivo by transplantation studies. Initial studies involving characterization of BM-MSCs derived from MDS based on morphology, expression of cell surface markers and differentiation have shown no difference when compared to MSCs derived from normal marrow [8]. Co-culture studies of BM MSCs obtained from MDS patients with hCD34+ cells from healthy donors have shown the diminished capacity of MDS-MSCs to support hCD34+ HSCs in long-term culture initiating cell assays [9, 10]. MDS-MSCs have also been reported to have structural chromosomal changes [11, 12]. Gene expression studies of MDS-MSCs have reported the altered expression of mediators of interactions with HSCs, including *osteopontin*, *Jagged-1*, *Kit-ligand*, *VEGF*, *TNFα* and *angiopoietin* [10, 13–15]. Recently Santamaría et al., have reported downregulation of *DICER1* and *DROSHA* gene and protein in MDS MSCs leading to impaired microRNA biogenesis [16].

Taken together, these observations provide a basis for the possible role of BM-MSCs in the pathophysiology of MDS. In this study, we have conducted comparative analyses of phenotypic and molecular changes between C-MSCs and those derived from MDS-MLD patients.

Materials and Methods

Isolation and Culture of Bone Marrow MSCs

Fresh or cryopreserved mononuclear cells were seeded at approximately 1×10^5 cells/cm^2 in a T75 flask containing alpha minimum essential medium (αMEM) supplemented with 1 mM L-glutamine, 5% human platelet lysate (HPL) and 1% penicillin/streptomycin. MSCs were cultured and expanded as previously described [17].

Immunophenotypic Analysis

MDS-MSCs and C-MSCs were analyzed using flow cytometry by criteria established by the ISCT [1]. Cultured MSCs were analyzed for surface expression by flow cytometry (FACS Calibur; BD Biosciences Pharmingen, San Diego, CA, USA) using Cell Quest software (BD Biosciences Pharmingen, San Diego, CA, USA). The MSCs were characterized using flow cytometry-based positive reaction for surface CD29, CD73, CD90, CD105, CD146, and CD271 and negative for CD34, CD45 and CD14 (BD Biosciences Pharmingen, San Diego, CA, USA) [17] with appropriate isotypic controls.

Differentiation Analysis

To evaluate the differentiation potential of in vitro cultured MSCs, cells were differentiated to adipogenic and osteogenic lineage as described previously [18]. Adipogenic and osteogenic differentiation was assessed by Oil O Red and Alizarin stains respectively.

Apoptosis Analysis

Apoptosis analysis was carried out by following the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA, USA). In vitro, cultured MDS-MSCs and C-MSCs at P4 were stained with Annexin-V, and 7-AAD analyzed in a flow cytometer. Data were acquired in BD Flow Aria III (BD Biosciences Pharmingen, San Diego, CA, USA) and analyzed using FlowJo software (BD Biosciences Pharmingen, San Diego, CA, USA). Early apoptotic cells were defined as Annexin-V-positive, 7-AAD-negative cells; late apoptosis cells were identified as Annexin-V and 7-AAD double positives (Fig. 3b). For calculation of the percentage of total apoptotic cells, early and late apoptotic cells were summed up [18].

Cell Cycle Analysis

For cell cycle analysis 3–5 $\times$ 10^5 BM-MSCs, were fixed overnight at 4 °C in 4% paraformaldehyde (Sigma-Aldrich) and followed by permeabilization with 0.2% TritonX-100. Cells were further stained with FITC conjugated-Mouse Anti-Human Ki67 Set (BD Biosciences Pharmingen, San Diego, CA, USA) according to manufacturer's instruction and 0.1ug/ul DAPI. Data were acquired in BD Flow Aria III (BD Biosciences Pharmingen, San Diego, CA, USA) and analyzed using FloJo software (BD Biosciences Pharmingen, San Diego, CA, USA) [19].

On the basis of differences in Ki-67 expression level and DNA content of cells, resting/quiescent (G0) population can be distinguished from other proliferating cells (G1, S, G2/M phases). Generally, the G0 cells have lower levels of Ki-67 and DNA levels so that these cells may be distinguishable from other proliferating cells. Ki-67-FITC signal is acquired in logarithmic mode and DAPI signal in linear mode. Gating of Go contour of DNA content (x-axis) vs. Ki67-Ag immunoreactivity (y-axis) is done to obtain a percentage of cells in G0, G1, S, G2/M phases.

Gene Expression Analysis

Quantitative real-time PCR was performed as described in earlier studies [13]. Briefly, total RNA was prepared from the in vitro cultured mesenchymal stromal cells, in TriZol

(Thermo Fisher Scientific, MA, USA) using conventional protocols for RNA preparation. Following quantification of RNA amount, cDNA synthesis was done using a High Capacity Reverse Transcription Kit (Applied Biosystems, MA, USA). RT-PCR reactions were performed using DyNAmo Flash SYBR Green qPCR Master Mix (Thermo Scientific, MA, USA), and qPCR data were normalized against beta-actin.

Karyotyping

Karyotyping of MSCs was carried at passages 4–5 to verify the chromosomal integrity. Metaphase chromosomes were prepared using standard protocols at a 400–550 GTG band level. Axioskop microscope (Zeiss, Oberkochen, Germany) was used to capture chromosome images which were analyzed on the Ikaros software (MetaSystems, Altlussheim, Germany).

Statistical Analysis

GraphPad Prism 5.00 (GraphPad Software, San Diego, CA) was used to perform statistical analysis and for graphing the data. Human data were analyzed using the Mann–Whitney U-test with 95% CI. A two-tailed P value < 0.05 was considered statistically significant. All data are expressed as mean (SEM).

Results

Patients and Bone Marrow Samples

Bone marrow aspirates from diagnostic samples of MDS-MLD (n = 6, median age 53 years, range 42–58 years) and control (n = 6, median age 56.5 years, range 32–65 years) were collected after informed consent in accordance with the institutional review board of Christian Medical College (CMC), Vellore. Control BM samples were obtained from individuals with morphologically normal marrow in patients with diseases where no known abnormalities exist in the hematopoietic stem cell or the stroma.

Morphology

While both MDS-MSCs (n = 6) and C-BM-MSCs (n = 6) exhibited normal fibroblast morphology in early passage (P1-P2), the former changed to abnormal cellular morphology after the initial passage (P1-P2) from typical fibroblast-like morphology (Fig. 1a) to larger, flat, comparatively fewer cells and appearance of these cells suggestive of cellular senescence (Fig. 1b).

Immunophenotyping Studies

Sufficient numbers of MDS-MSCs, obtained from six samples (P2) were of $> 90\%$ positive for CD73, CD90 and CD105 and negative ($< 1\%$) for CD34, CD45, and CD14 surface markers (Fig. 2a). This was similar to the C-MSCs. Additionally, MDS-MSCs (P3) from six patients and C-MSCs were characterized based on cell surface expression of CD146 and CD271 (Fig. 2b). There was no significant change in frequency of CD146+ MSCs (single positive), CD271+ MSCs (single positive) and CD146+ CD271+ (double positive) MSCs within the total MSC population in MDS compared to C-MSCs (Fig. 2b).

Adipogenic and Osteogenic Differentiation

The ability of MDS-MSCs and C-MSCs to undergo adipogenic and osteogenic differentiation was comparable (Fig. 3a).

Assessment of Apoptosis and Cell Proliferation Studies

The apoptosis assay showed sevenfold increase in the percentage of apoptotic cells in MDS-MSCs at P4 ($0.2667 \pm 0.07535\%$, $n = 3$; $P < 0.05$) compared to C-MSCs at P4 ($0.03633 \pm 0.01802\%$, $n = 3$; $P < 0.05$) (Fig. 3c). To analyze the proliferation status of MDS-MSCs, Ki-67 expression was quantified by flow cytometric analysis (Fig. 4a). MDS-MSCs at P4 ($32 \pm 3.7\%$; $n = 3$; $P < 0.05$) had significantly lower percentage of proliferating cells compared to C-MSCs at P4 ($60 \pm 9.2\%$; $n = 3$; $P < 0.05$) (Fig. 4b).

Cell Cycle Studies

Cell cycle status of C-MSCs (n = 3) and MDS-MSCs (n = 3) samples at P4 were quantified using Ki67 and DAPI staining by flow cytometry. C-MSCs has significantly higher number of cells in G0 phase ($0.6400 \pm 0.1007\%$) than MDS-MSCs ($0.03633 \pm 0.01802\%$, $P < 0.005$; $n = 3$) (Fig. 4c). While C-MSCs were significantly lower in S-G2-M phase ($60.03 \pm 1.894\%$, $P < 0.005$; $n = 3$) than MDS-MSCs (79.33 ± 2.080, $P < 0.005$; $n = 3$) (Fig. 4c). Furthermore, G1 phase analysis showed C-MSCs were significantly higher in G1 phase (39.53 ± 1.235, $P < 0.05$; $n = 3$) than MDS-MSCs (24.03 ± 2.941, $P < 0.05$; $n = 3$) (Fig. 4c). These results indicate that MDS-MSCs display significant changes in cell cycle kinetics.

For apoptosis and cell cycle analysis initially, six samples were processed for these assays, but no quality data could be obtained from three MDS samples, owing to cell

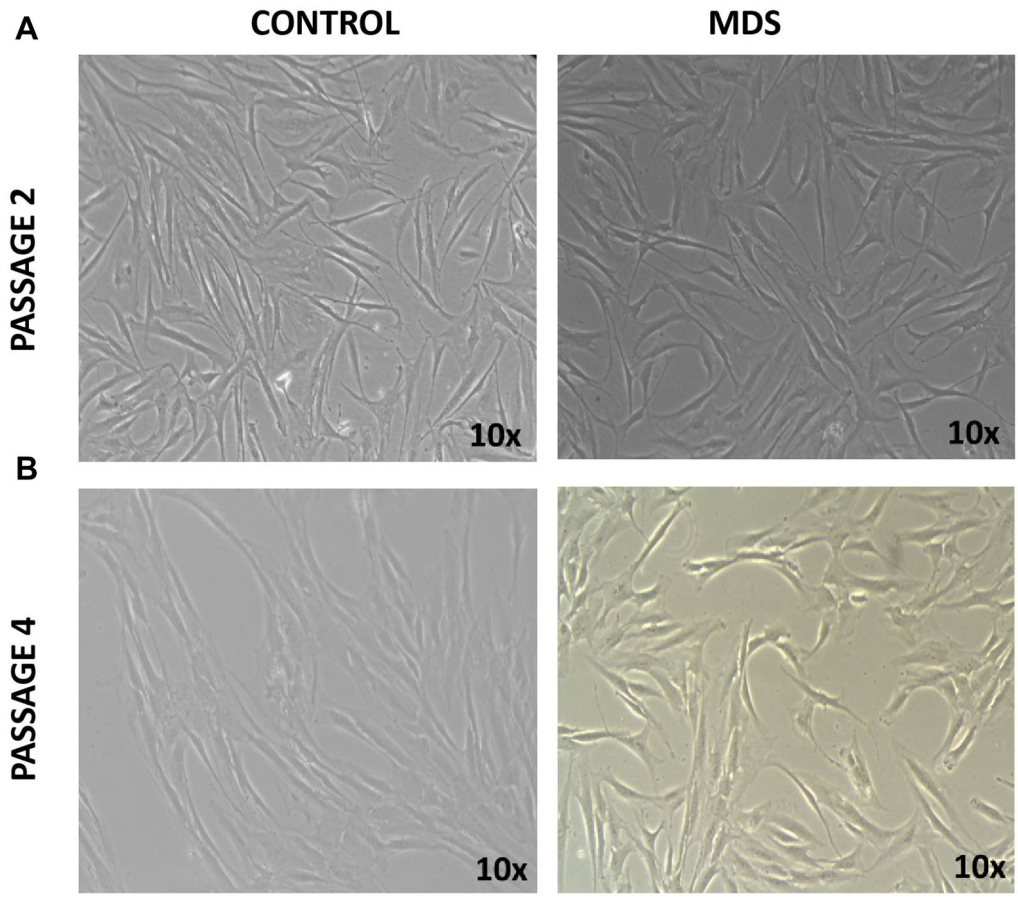

Fig. 1 Morphology of BM-MSCs from control and MDS (n = 6, P 2–4). Magnification ×10

loss. Later on, we attempted to revive cryopreserved samples. Unfortunately, we could not get confluent MSC cultures from these three MDS samples. Thus, we could provide apoptosis and cell cycle data for only three MDS samples.

Karyotyping

Cytogenetic characteristics of clinical samples showed abnormal karyotypes in four patients and normal karyotypes in two. The abnormalities seen were as follows: loss of Y (two patients), complex karyotypes (two patients). But MDS-MLD patients with abnormal BM karyotype did not show any abnormal karyotype in their MSCs.

Gene Expression Studies

Gene expression analysis of *CXCL12A, SCF, VEGF, ANGPT* and *LIF* in MDS-MSCs (n = 6) and compared them to C-MSCs (n = 6) showed a trend towards down-regulation of *VEGF, SCF,* and *ANGPT* expression in MDS-MSCs when compared to C-MSCs (Fig. 5a). There was no change in expression of *CXCL12A* and *LIF* between control and MDS-MSCs (Fig. 5a). Similarly, quantifying the mRNA expression levels of Notch signaling ligands (*JAGGED-1* and *DELTA-LIKE-1*), receptors (*NOTCH1, NOTCH3*) and downstream gene (*HES1*) showed that mRNA expression of JAGGED-1 had increased about two-fold in MDS-MSCs (n = 6) compared to C-MSCs (n = 6). The mRNA expression of *NOTCH-3*and *DELTA-LIKE-1* was downregulated about three-fold and sixfold, respectively in MDS-MSCs compared C-MSCs (Fig. 5b). Furthermore, we sought to determine whether any of the Wnt antagonist secreted by MSCs might be involved in it. Q-PCR was performed to quantify the mRNA expression levels of Wnt signaling inhibitory ligands (*DKK1 and DKK-2*) in MDS-MSCs (n = 6) and compared to C-MSCs (n = 6). The results showed that an approximately three-fold increase in mRNA expression of *DKK1* and a near two-fold increase in *DKK2* in MDS-MSCs (N = 6) compared to C-MSCs (n = 6) (Fig. 5c). While the mean values were different, there was a large inter-individual difference.

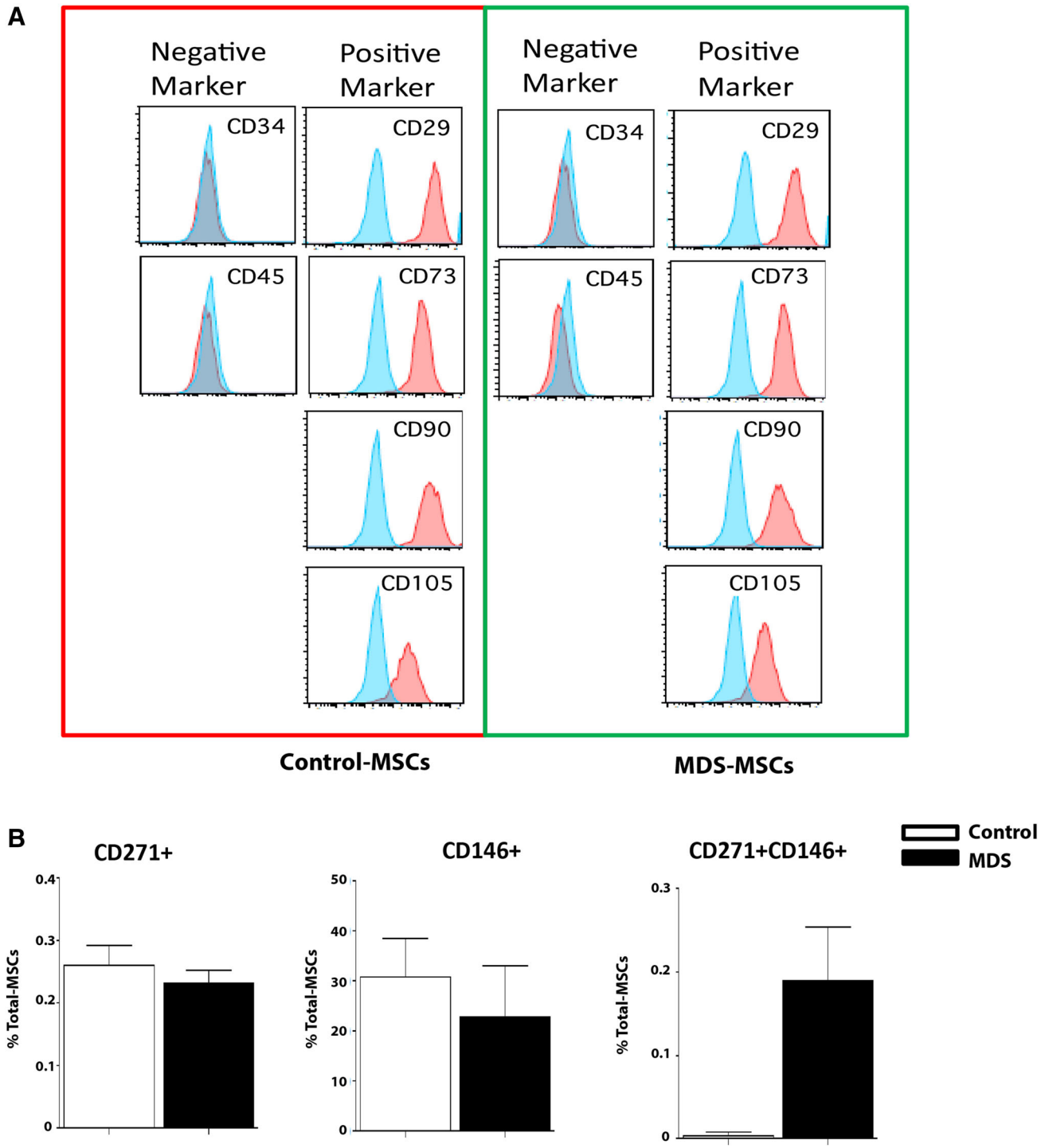

Fig. 2 **a** Phenotypic characterization of BM-MSCs from control and MDS (n = 6, P 3–4), **b** Quantification of cultured MSCs based on CD271 and CD146 expression (n = 6, P 3–4). $^{**}P < 0.05$

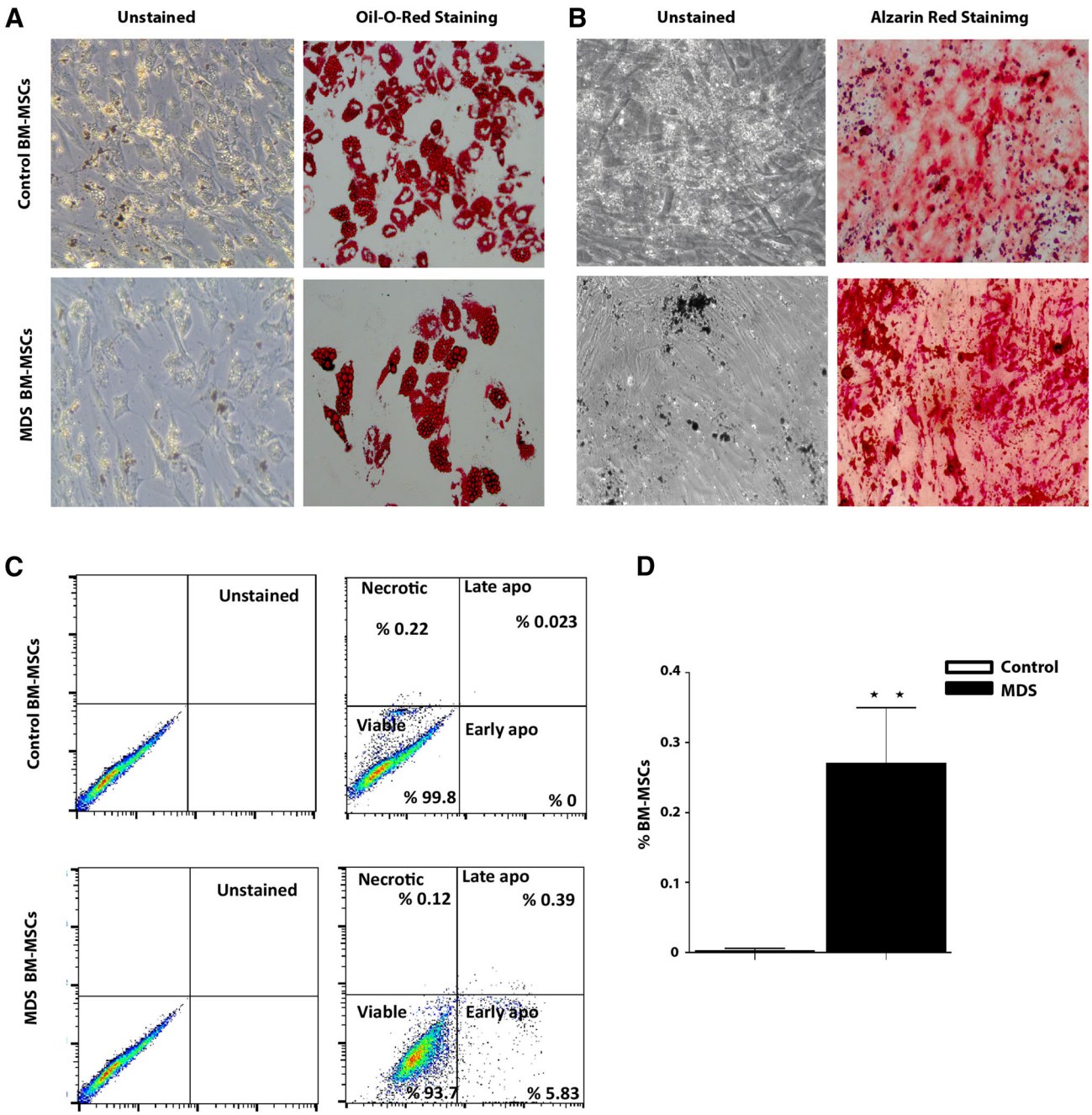

Fig. 3 **a** Adipogenic and osteogenic differentiation potential of MSCs from control and MDS cultured BM-MSCs (n = 6, P 3–4), **b** representative flow cytometry based apoptosis assay of MSCs from control and MDS cultured BM-MSCs (n = 3, P 3–4), **c** quantification of apoptotic cells at P4, n = 3. $^{**}P < 0.05$

Discussion

This study of MDS-MSCs augments our earlier report showing quantitative and qualitative changes in the de novo hematopoietic stem and its associated niche [20]. We conducted a comparative analysis of cultured MDS-MSCs from MDS-MLD patients and age-matched controls. In agreement with previous studies, MDS-MSC displayed an altered morphology and disrupted colony architecture during advanced passages beyond P3 [10, 13]. They also had a significantly lower percentage of proliferating cells compared to C-MSCs. Previous studies have shown that MSCs from patients across all MDS subtypes have significantly reduced proliferative potential in culture, which generally is accompanied by premature replicative senescence [10]. While some studies have shown MDS-MSCs to

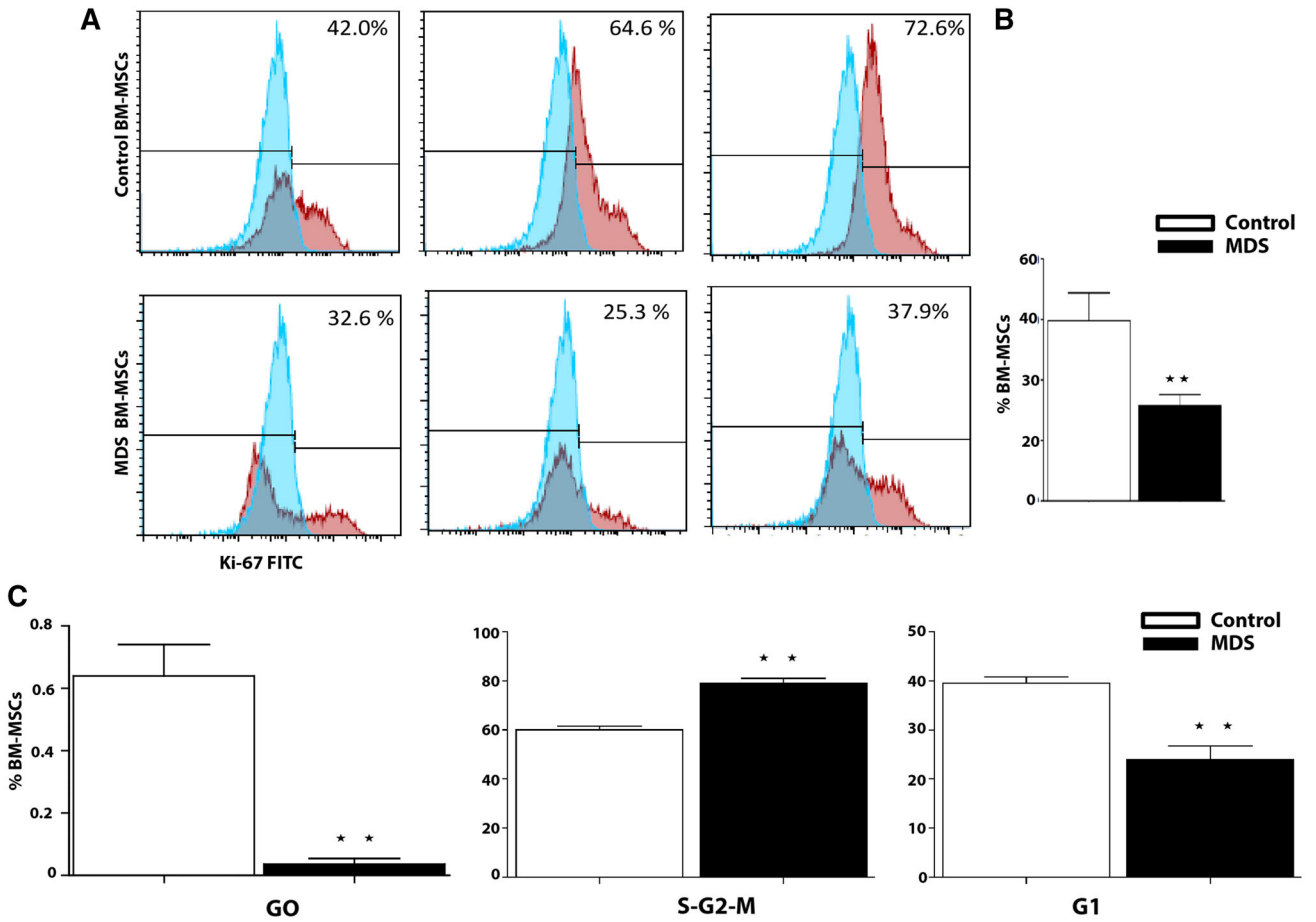

Fig. 4 Proliferation status of in vitro cultured MDS and control MSCs. **a** Percentage of RCMD and control MSCs showing positive staining for Ki67 antibodies. **b** Graphical representation of Flowcytometry data representing a percentage of Ki67 positive MSCs in MDS sample (n = 3) compared to control MSCs (n = 3). $^{**}P < 0.05$

have structural chromosomal changes suggesting that genetic alterations in MSCs trigger leukemogenesis [11, 12]. However, in this study karyotyping analysis of six MDS-MSCs showed normal karyotype in four samples and two samples could not be analyzed due to failure to obtain enough metaphase cells for karyotyping. Abnormal MSC proliferation in low-risk MDS has been attributed to inactivation of focal adhesion kinase (FAK) [15]. Zhao et al. have shown that the down-regulation of Dicer1 in MSCs derived from MDS patients contributes to their early senescence and impaired support for normal hematopoiesis [16]. These changes in the cell cycle and proliferative capacity of MDS BM-MSCs further supports the notion that MDS-MSCs are part of the abnormal BM stroma in MDS [12, 21]. Whether these changes are the cause or effect of the hematopoietic changes will need further evaluation.

Using MSCs specific cell surface receptor antibodies (positive for CD73, CD90, and CD105 and negative for CD34, CD45, and CD14 antibodies), we could not detect any significant phenotypic changes in cultured MDS-MSCs and C-MSCs [1]. Also, there was no significant change in the frequency of single positive CD146+ MSCs, single positive CD271+ MSCs and double positive CD146+ CD271+ MSCs within the total cultured MSCs in C-MSCs and MDS-MSCs. Thus, in addition to CD146 and CD271, additional unique cell surface molecule should be identified for quantitatively analyzing the phenotypic changes in MDS-MSCs.

Differences were noted in the apoptosis patterns of MDS-MSCs. The apoptosis assay performed by Annexin V and 7AAD results showed that the percentage of apoptotic cells was significantly higher in MDS-MSCs compared to the C-MSCs ($P < 0.05$). The basis for this difference is unclear but may be due to exposure to niche derived TNFα in bone marrow milieu of these MDS patients [17]. It has been demonstrated TNFα triggers pro-apoptotic and proinflammatory signals in MDS stroma cells, leading to an auto-amplification loop, where these complex signals from

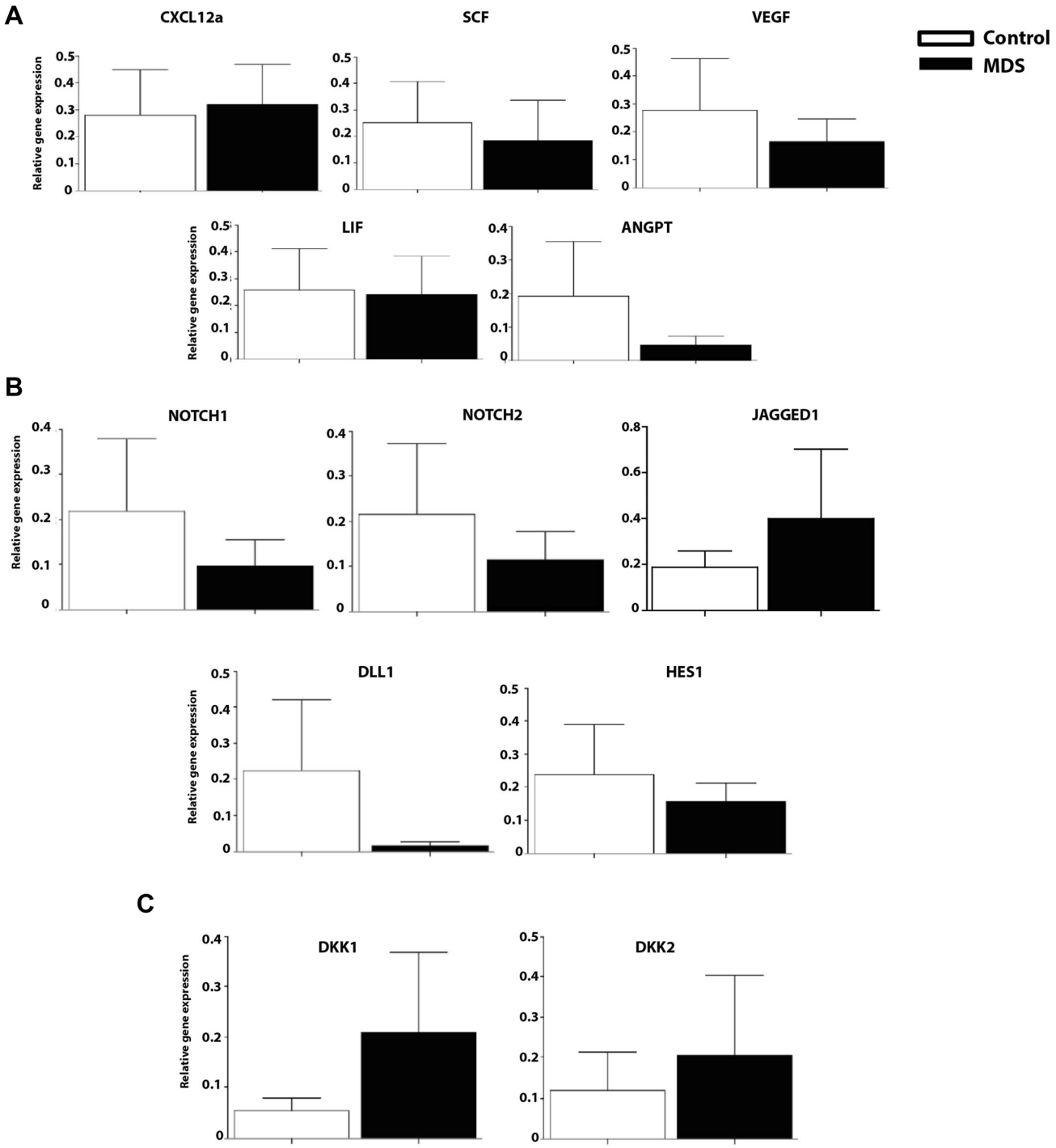

Fig. 5 **a** Relative cytokine gene expression analysis **b** Relative mRNA expression of Notch signaling ligands, their corresponding receptors and downstream genes, **c** relative mRNA expression of Wnt signaling inhibitors in MDS MSCs and control MSCs (n = 6, P 3–4)

stromal cells interact with malignant hematopoietic clones in MDS [18].

Along with the phenotypic changes as observed in MDS-MSCs, we analyzed the expression level of cytokines in MDS-MSCs. Some of cytokines and growth factors like IL-6, Flt3-L (FL), SCF, G-CSF, LIF, M-CSF, GM-CSF, TPO, CXCL-12 (SDF1) and IL-11 have been shown to be secreted by MSCs. These molecules act as physiological mediators between MSCs and HSPCs [22, 23], and therefore it is possible that aberrant expression of these molecules may contribute to the insufficient stromal support for HSPCs. Specifically, we found a diminished expression of

VEGF, *SCF,* and *ANGPT* in MDS-MSCs. Mice lacking expression of SCF in BM stromal cells have been shown to present macrocytic anemia [21], a feature in many patients with MDS. Similarly, MSCs from both low risk [22] and high-risk MDS patients [23] exhibited reduced expression of ANGPT, a cytokine responsible for the maintenance of HSPC quiescence [24]. These abnormal MDS-MSCs may not be capable of supporting normal hematopoiesis and may influence clonal propagation [10, 24].

Similarly, the critical role of Notch and Wnt signaling between MSCs and HSPCs in the context of MDS pathophysiology has been extensively studied [25, 26]. Among all genes related to Notch signaling tested in this study, we observed altered expressions of *JAGGED1, HES1, DLL1, NOTCH, and NOTCH2* in MDS-MSCs compared to C-MSCs. Increased expression of *JAGGED1* in MDS-MSCs is agreement with reported MDS mouse model wherein the active expression of *JAGGED-1* in niche cells lead to consequent activation of Notch signaling in hematopoietic cells [27]. The above data show that MDS-MSCs have dysregulated Notch signaling which may contribute to the pathogenesis of MDS. Further studies in different types of MDS in larger numbers are needed to confirm this possibility.

In this study, quantitative mRNA expression level of Wnt signaling inhibitory ligands (*DKK1 and DKK2*) results showed that *DKK1 and DKK2* expression was increased in MDS-MSCs compared to C-MSCs. Overexpression of *DKK1* in stromal cells has been shown to increase the proliferation rate of HSCs, rendering them incapable of reconstituting hematopoiesis upon serial transplantation [28]. Also, inhibition of canonical Wnt signaling pathway by Wnt inhibitory ligands was attributed to the diminished proliferative capacity of MSCs derived from MDS patients [25, 26].

Taken together, our comprehensive analysis of cultured MSCs from patients with MDS-MLD has shown that they have phenotypic and molecular alterations leading functional changes. Whether these changes in the stroma are the cause or consequence of the abnormal hematopoiesis in these patients remains unclear. Evaluating larger numbers of patients with serial samples, if possible, will provide further useful data. RNA-seq, co-culture studies, and transplantation in appropriate transgenic models will also help clarify the origin and implications of these changes. Such data could then identify potential targets for therapeutic intervention.

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Compliance with Ethical Standards

Conflict of interest Authors declares that they have no conflict of interest.

Ethical Standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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