Impaired expression of DICER, DROSHA, SBDS and some microRNAs in mesenchymal stromal cells from myelodysplastic syndrome patients

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ABSTRACT

Background

Recent findings suggest that a specific deletion of Dicer1 in mesenchymal stromal cells-derived osteoprogenitors triggers several features of myelodysplastic syndrome in a murine model. Our aim was to analyze *DICER1* and *DROSHA* gene and protein expression in mesenchymal stromal cells (the osteoblastic progenitors) obtained from bone marrow of myelodysplastic syndrome patients, in addition to microRNA expression profile and other target genes such as *SBDS*, a *DICER1*-related gene that promotes bone marrow dysfunction and myelodysplasia when repressed in a murine model.

Design and Methods

Mesenchymal stromal cells from 33 bone marrow samples were evaluated. *DICER*, *DROSHA* and *SBDS* gene expression levels were assessed by real-time PCR and protein expression by Western blot. MicroRNA expresion profile was analyzed by commercial low-density arrays and some of these results were confirmed by individual real-time PCR.

Results

Mesenchymal stromal cells from myelodysplastic syndrome patients showed lower *DICER1* (0.65±0.08 *vs.* 1.91±0.57; *P*=0.011) and *DROSHA* (0.62±0.06 *vs*. 1.38±0.29; *P*=0.009) gene expression levels, two relevant endonucleases associated to microRNA biogenesis, in comparison to normal myelodysplastic syndrome. These findings were confirmed at protein levels by Western blot. Strikingly, no differences were observed between paired mononuclear cells from myelodysplastic syndrome and controls. In addition, mesenchymal stromal cells from myelodysplastic syndrome patients showed significant lower SBDS (0.63±0.06 *vs*. 1.15±0.28; *P*=0.021) gene expression levels than mesenchymal stromal cells from healthy controls. Furthermore, mesenchymal stromal cells from myelodysplastic syndrome patients showed an underlying microRNA repression compared to healthy controls. Real-time PCR approach confirmed that mir-155, miR-181a and miR-222 were down-expressed in mesenchymal stromal cells from myelodysplastic syndrome patients.

Conclusions

This is the first description of an impaired microRNA biogenesis in human mesenchymal stromal cells from myelodysplastic syndrome patients, where *DICER1* and *DROSHA* gene and protein downregulation correlated to a gene and microRNA abnormal expression profile, validating the animal model results previously described.

Key words: mesenchymal stem cells, *DICER*, *DROSHA*, myelodysplastic syndrome.

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The online version of this article has a Supplementary Appendix.

Introduction

In recent years, several studies have reported that the BM microenvironment in MDS shows qualitative and functional abnormalities *in vitro*,[1,2] suggesting a potential role for these cells in inefficient or malignant hematopoiesis.[3] However, the study design of most these studies was rather complex structure and results are controversial.

Mesenchymal stromal cells (MSC) are a small, non-hematopoietic, BM microenvironment cell population. Considered to be the osteoblastic progenitors, they are also enrolled as key components into the hematopoietic microenvironment.[2] Some authors[2,4-7] have shown that MSC display several morphological, immunophenotypic and genetic alterations in these syndromes. In a previous study,[8] we observed that MSC from MDS patients show several genomic alterations with their consequent loss of function. However, the effect of these abnormal features on the impaired development of HSC remains unclear.

More recently, Raaijmakers *et al.*[9] reported that deletion of *Dicer1*, an RNase III enzyme involved in microRNA biogenesis, in MSC-derived osteoprogenitors resulted in peripheral blood cytopenias, myelodysplasia and secondary leukemia in a murine model, providing evidence that specific molecular alterations in the microenvironment could result in clonal hematopoiesis. Furthermore, they observed a reduced expression of *SBDS*, the gene mutated in Schwachman–Bodian–Diamond syndrome, related to bone marrow failure and with high risk of developing leukemia.[9] The aim of the present study was to analyze *DICER1* and *DROSHA* (another RNA III endonuclease) gene and protein expression in MSC (the osteoblastic progenitors) obtained from BM of MDS patients, as well as the microRNAs expression along with some target genes such as *SBDS*, *TP53*, *PTEN*, *MYC* and *SDF1*, showing that MSC from MDS patients showed a reduced expression of *DICER1*, *DROSHA* and *SBDS* when compared to normal MSC.

Design and Methods

Patients and control samples

For *DICER1* and *DROSHA* analysis, 33 BM samples from *de novo* and untreated MDS, (8 5q- syndrome, 5 RA, 5 RARS, 8 RCMD, 4 RAEB, 2 MDS-U and 1 hypocellular MDS) were included. MDS diagnosis was based on the 2008 WHO criteria.[10] Male to female ratio was 14:19 and mean age was 73.9 years (range 53-87). Non-malignant BM samples were obtained from 25 healthy donors (HD), 12 men and 13 women, with a mean age of 75.6 years (range, 55-87). Additionally, paired mononuclear cells (MNC) from 11 MDS and 8 HD were obtained. The study was approved by the institutional review board at each participating center and all patients gave written informed consent to use biological samples and clinical data.

Isolation and expansion of mesenchymal cells

MSC were isolated and characterized by flow cytometry from BM samples of patients and healthy controls as previously described.[8,11] In addition, osteogenic, adipogenic and chondrogenic differentiation was demonstrated following standard procedures described elsewhere.[8,12]

Gene expression analysis

Total RNA was obtained from either MSC or MNC with Trizol (Invitrogen) and subsequent reverse transcription was carried out using the High Capacity kit (Applied Biosystems, Foster City, CA, USA). Gene expression of *DICER1*, *DROSHA*, *SBDS*, *TP53*, *SDF1*, *MYC*, *PTEN* and *GAPDH* (as control gene) was quantified by using commercial TaqMan® Gene Expression Assays and the Step One Plus Real-Time PCR System (Applied Biosystems). Relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ values where:

$\Delta Ct = Ct_{Gene} - Ct_{GAPDH}$ and $\Delta\Delta Ct = mean(\Delta Ct_{MDS}) - mean(\Delta Ct_{HealthyDonors})$.

Values were expressed as mean ± standard error. Differences between MDS and controls were analyzed using non-parametric Mann-Whitney U test and Wilcoxon's test for paired samples.

microRNA expression analysis

RNA (350-1000 ng) of MSC from 21 low-risk MDS and 11 HD were retrotranscribed with Megaplex™ RT Primers primer pool (Applied Biosystems). We loaded 384-well microfluidic cards (TaqMan® MicroRNA Array A) with retro-transcription product and PCR runs were performed on a 7900HT Fast Real-time PCR system (Applied Biosystems).

MicroRNA expression data were processed within the R statistical computing environment (version 2.10.0), using *ΔΔCt* standard procedures from the *HTqPCR package*.[13] The raw Ct values were normalized using the array endogenous control features by $\Delta Ct_{miRNA} = Ct_{miRNA} - mean(Ct_{MammU6})$. MicroRNA differential expression was tested based on the ΔΔCt values where:

$\Delta\Delta Ct_{miRNA} = mean(\Delta Ct_{MDS}) - mean(\Delta Ct_{HealthyDonors})$.

The *Benjamini-Holm* method was applied for multiple testing corrections of raw P values extracted from the t-test statistic. Also the Log Transformed Relative Quantifications ($2^{-\Delta\Delta Ct}$) for each microRNA, the so called Fold Changes (FC), were calculated.

To confirm microRNA expression findings by low-density arrays, we performed individual quantitative PCR for the following microRNAs: Hsa-mir-26b, Hsa-mir-34c-5p, Hsa-mir-125a-5p, Hsa-mir-129-3p, Hsa-mir-146a, Hsa-mir-150, Hsa-mir-153, Hsa-mir-155, Hsa-mir-181a and Hsa-mir-222. RNA samples used in low-density arrays from 19 MDS patients (6 5q-, 1 RA, 1 RARS, 8 RCMD, 2 MDS-U and 1 hypocellular MDS) and 8 HD were retrotranscripted according to TaqMan® MicroRNA Reverse Transcripcion Kit (Applied Biosystems). Fold-Differences were calculated using the same *ΔΔCt* method described previously.

Data relating to the targeting of our miRNAs over genes were collected from the *miRNA* Validated Target repository, available at the miRWalk database (*web link http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/mirnatargetpub.html*).

For a better biological understanding of the expression results, we collected the genes reported as validated targets of our relevant microRNAs from the miRWalk database (data released March 2011).[14] Furthermore, their gene functional pathways were analyzed using the Ingenuity Pathways Analysis software (IPA).

Western blot analysis

Whole cell lysates were obtained from MSC. Samples (30 µg) were loaded on a 6% and 10% SDS-PAGE gel for antibody SBDS. Used primary antibodies were: mouse α-DICER1 (1:500; ab14601; Abcam Inc.), rabbit α-DROSHA (1:2000; ab12286; Abcam Inc.), goat α-SBDS (1:200, sc-49257; Santa Cruz Biotechnology, Inc.) and rabbit α-calnexin (1:50000; SPA-860; StressGen) as loading control. Membranes were incubated with either anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). Specific bands were visualized by using ECL Western Blotting Detection Reagents (Amersham Biosciences).

Results

DICER1 *and* DROSHA

Gene expression analysis of *DICER1* and *DROSHA* by real-time PCR in MSC from MDS patients showed significantly lower *DICER1* (0.65±0.08 *vs*. 1.91±0.57; *P*=0.011) and *DROSHA* (0.62±0.06 *vs.* 1.38±0.29; *P*=0.009) (Figure 1A) gene expression levels in MSCs from MDS patients when compared to healthy donors (HD-MSC). By contrast, no difference in *DICER1* (1.35±0.29 *vs*. 1.19±0.27; *P*=0.9) or *DROSHA* (1.48±0.10 *vs*. 1.12±0.22; *P*=0.09) gene expression was observed between MNC from MDS and HD (Figure 1B). These results were independent of leukocyte differential counts in original samples. When MSC were compared with MNC from paired MDS patients (n=11) a significantly lower expression of both *DICER1* (0.19±0.03 *vs.* 1.36±0.34; *P*=0.003) and *DROSHA* (0.87±0.11 *vs.* 1.48±0.12; *P*=0.008) were observed in stromal cells (Figure 1C). When MSC and MNC from healthy donors (n=8) were compared, no differences were observed in either *DICER1* (MNC 1.19±0.77 *vs*. MSC 1.91±2.84; *P*=0.4) or *DROSHA* (MNC 1.12±0.61 *vs*. MSC 1.39±1.45; *P*=0.6) gene expression levels. Finally, we assessed the association between RNA levels of both markers in MSC, resulting in a strong correlation between *DICER* and *DROSHA* gene expression levels (Spearman's Rho correlation=0.832, *P*<0.001, Figure 1D).

Because 5q- syndrome patients are a separate and well-defined entity among MDS, we compared *DICER1* and *DROSHA* expression between 5q- syndrome and other MDS but no statistical differences were observed (*P*>0.05).

In order to confirm quantitative PCR data, Western blot analysis was performed in MSC from MDS and compared with controls. MSC from MDS patients showed a lower protein expression of DICER1 and DROSHA, supporting gene expression results (Figure 1A).

SBDS expression

Since *Dicer1*-deleted murine osteoprogenitors showed lower *Sbds* expression levels, and deletion of Sbds in mouse osteoprogenitors induced bone marrow dysfunction with myelodysplasia,[9] we decided to quantify *SBDS* in human MSC from MDS. *SBDS* was significantly down-expressed by real-time PCR analysis in MSC from MDS patients when compared with MSC from healthy donors (0.63±0.06 *vs*. 1.15±0.28; *P*=0.021) (Figure 2).

According to Western blot, different SBDS protein levels were observed among MSC from MDS patients (Figure 2B). Although MSC from non 5q- MDS groups (5 RCMD, 2 RAEB and 1 RARS) showed no clear differences compared to healthy MSC, we observed a reduced SBDS expression in 3 out of 4 MSC from 5q- in comparison to normal MSC, but the small number of evaluated samples

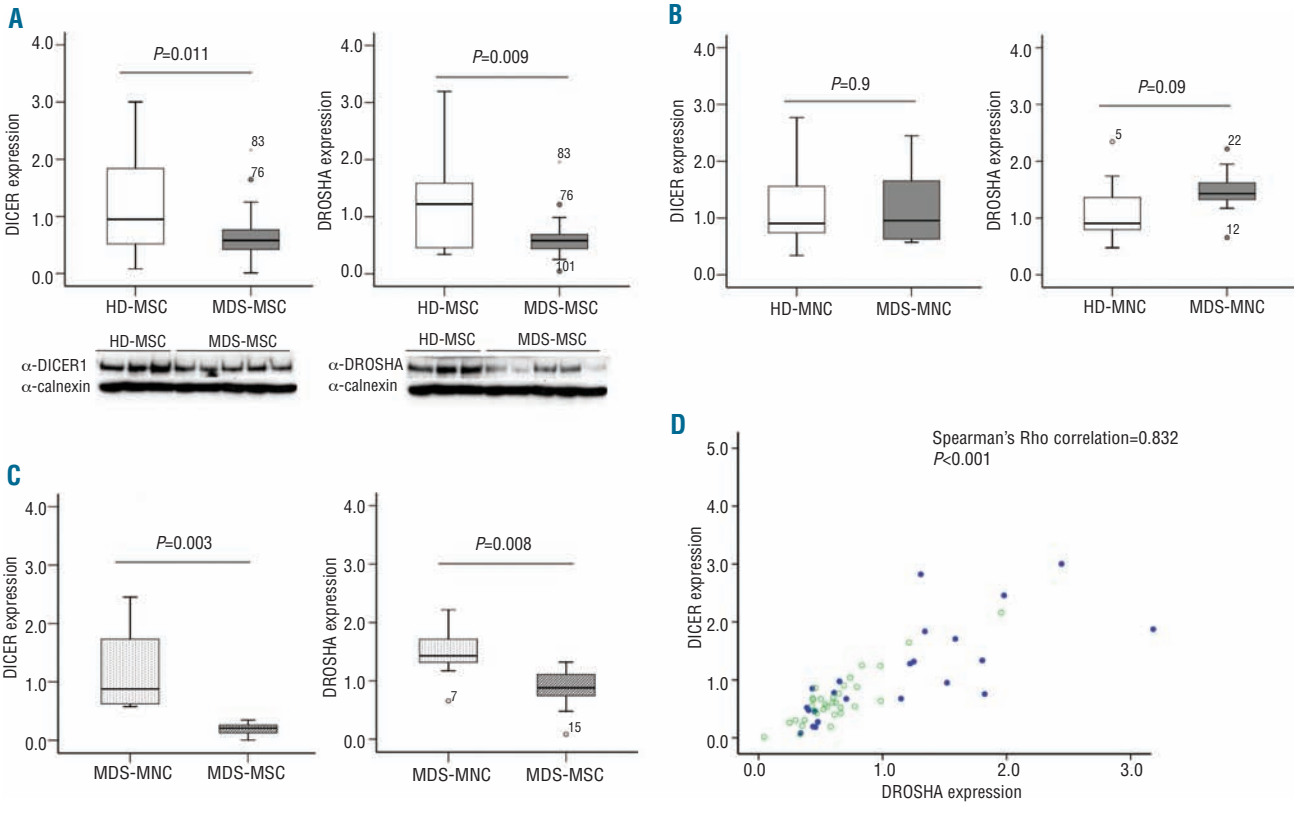

Figure 1. DICER1 and DROSHA expression in myelodysplastic syndrome patients (MDS) and healthy donors (HD). (A) Analysis in mesenchymal stromal cells (MSC) by quantitative PCR and Western blot. (B) Analysis in mononuclear cells (MNC) by quantitative PCR. (C) ***DICER1*** and ***DROSHA*** comparison between paired MSC and MNC from MDS patients by quantitative PCR. (D) Relationship between DICER and DROSHA in MSCs from MDS patients. Outlier's values in the boxplot are represented by a circle or asterisk (extreme outliers)

means results are not conclusive. Furthermore, no correlation between gene and protein expression were observed in those MDS cases with paired samples (n=8; *data not shown*).

MicroRNA expression analysis

When differential expression analysis of microRNA quantitative-PCR arrays was performed, an underlying microRNA repression in MDS samples compared to healthy controls was observed (Figure 3). Although the multiple testing adjusted *P* values did not reach statistical significance, we found 370 out of all 380 tested microRNAs (97%) with a decreased expression level in MDS cells *versus* control cells (averaged FC=-0.42); 159 out of those ranked microRNAs showed a raw P value of less than 0.05 (averaged FC=-0.71, minFC=-0.31, maxFC=-1.28; *Online Supplementary Table S1*).

Because *DICER1* and *DROSHA* were down-expressed, and a tendency towards lower expression in miRNAs was observed, an analysis of some miRNAs involved in hematopoiesis regulation was performed by real-time PCR. hsa-miR-155 (*P*=0.015) and hsa-miR-181a (*P*=0.05) were significantly down-expressed in MSC from MDS patients, whereas a trend towards lower hsa-miR-222 expression in MSC from MDS was also observed (*P*=0.082; Table 1). Those same microRNA were shortlisted among the 159 down-regulated miRNAs into the qPCR-array data with the corresponding FC values: hsa-miR-222 =-0.406; hsa-miR-181a =-0.597; hsa-miR-155 =-0.464 (*Online Supplementary Figure S1*).

DICER1 and *DROSHA* have been reported to be targets of these three miRNAs. In fact, 32 of 159 down-regulated microRNAs presented reported interactions with *DICER1* (*Online Supplementary Table S2*). Furthermore, many important genes involved in hematopoiesis were found as validated targets of the three down-expressed microRNAs. According to Ingenuity Pathways Analysis software, the main functional pathways in which they are involved are: cell death, cell growth and proliferation, cell cycle, cellular development, DNA replication and repair, cancer, hematologic system development and function, gene expression, cellular function and maintenance.

Furthermore, four additional target genes of down-expressed microRNA with relevant role in MDS pathogenesis (*MYC*,[15] *PTEN*,[16] *SDF1*[17] and *TP53*[18]) were also evaluated. Of these selected genes, only SDF1 showed a significant lower expression in MSCs from MDS patients compared to MSC from healthy donors (*P*=0.026; Table 2).

Discussion

In a previous study[8] we reported that MSC from MDS patients can show genomic changes with functional impairment, suggesting a possible role in MDS pathophysiology. Here, we describe for the first time how MSC from MDS patients show a low expression of *DICER1*, *DROSHA* and *SBDS*, as well as a global downregulation of microRNA expression.

Raaijmakers *et al.*[9] recently published a pivotal paper in which they reported that specific deletion of *Dicer1* in murine MSC-derived osteoprogenitors triggered blood cytopenias, high apoptosis rate, myelodysplasia and subsequent AML development. Based on these findings, we analyzed *DICER1* and *DROSHA*, two relevant RNA endonucleases, in MSC from MDS patients, and we observed a lower gene and protein expression levels of both of them when compared to healthy donors MSC. This low expression in MSC was also observed when paired mononuclear cell-MSC from MDS patients were evaluated. By contrast, no differences were observed between mononuclear cells from MDS and healthy donors or paired mononuclear cells-MSC from healthy donors. Therefore, our results show that DICER1 and DROSHA down-regulation is exclusively observed in MSC from MDS, confirming previous findings in murine models.

One of the most striking features in the study performed by Raaijmakers *et al.* was the reduced expression of *Sbds*, the gene mutated in Schwachman-Bodian-Diamond syndrome, a human entity characterized by BM failure and tendency to develop AML, in MSC from the murine MDS model.[9] In order to verify whether this gene could also be altered in human MDS we compared SBDS expression in MSC from MDS and healthy donors, showing that SBDS gene expression was down-expressed in the former group. But at a protein level, only 5q- syndrome samples showed a trend towards lower SBDS expression, suggesting that, at least in 5q- cases, SBDS downexpression in MSC could play a potential role in MDS pathogenesis.

DICER1 and *DROSHA* are involved in microRNA bio-

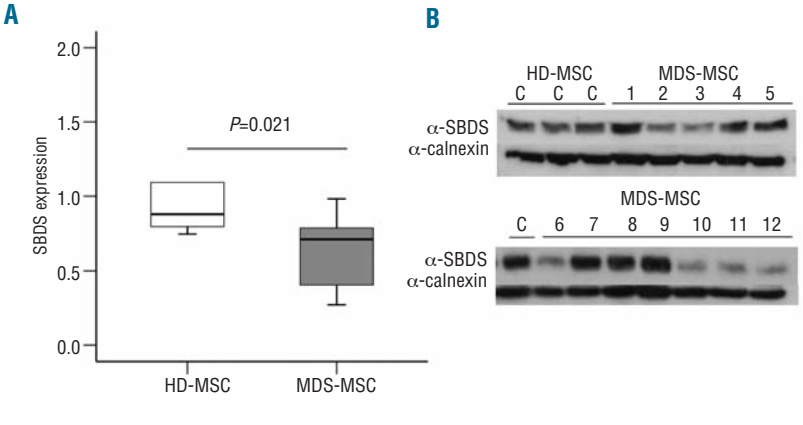

Figure 2. *SBDS* gene expression in mesenchymal stromal cells (MSC) from myelodysplastic syndrome patients (MDS) and healthy donors (HD). Control MSC from healthy donors are indicated with a C letter (3 samples in upper panel and 1 in lower panel). MSC from MDS samples are detailed as follows: 5q- syndrome (lines 2, 3, 6 and 8), RARS (lines 4, 5, 7, 11 and 12), RAEB1 (line 1), RAEB2 (line 10), and RARS (line 9).

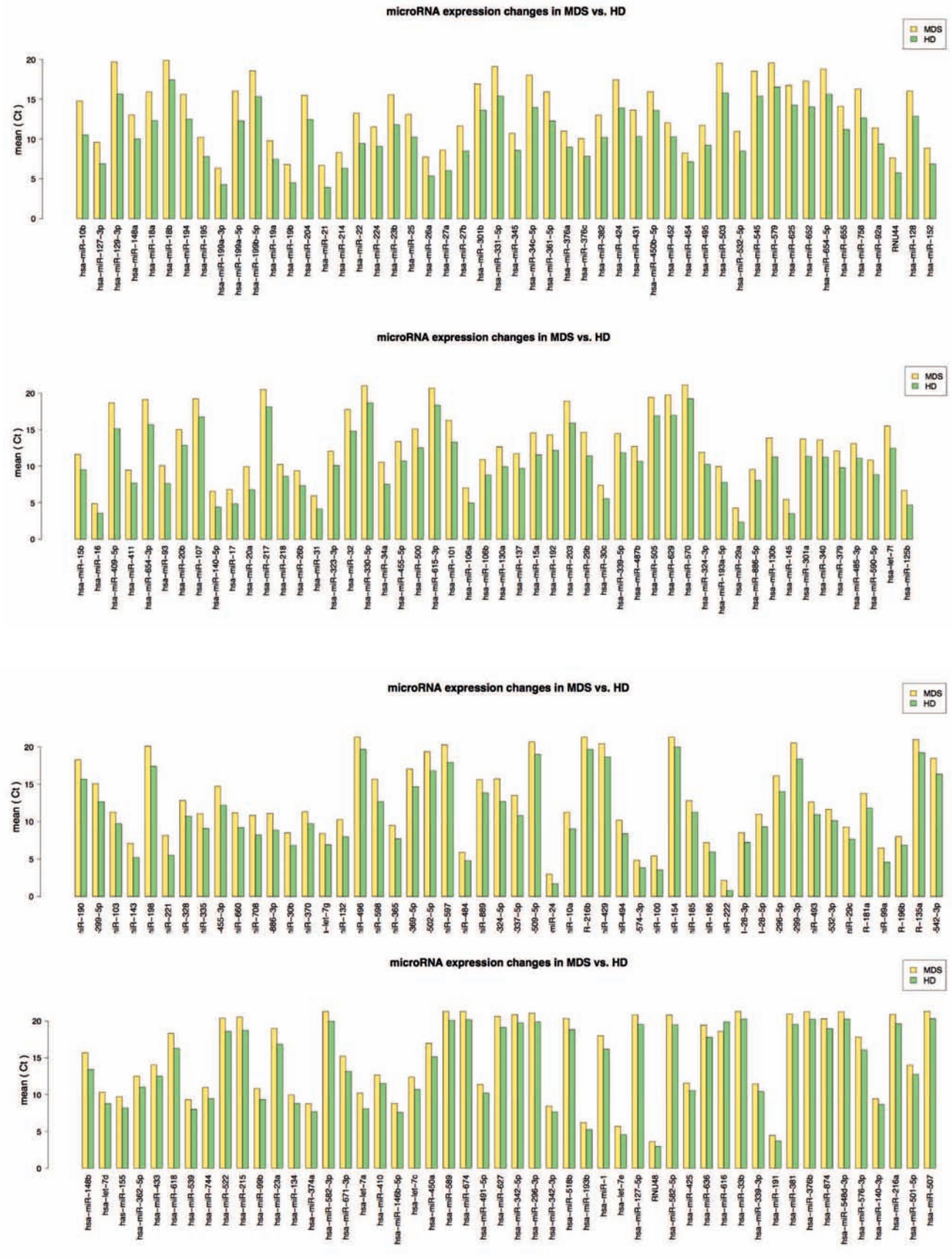

Figure 3. MicroRNA expression pattern by TaqMan® MicroRNA Arrays. A barplot representing the most 200 down-regulated MicroRNA is shown. Bars represent individual microRNA Ct mean values in MDS samples (in yellow) *versus* Ct mean values in HD samples (in green). Mean expression value from MDS and control samples for each evaluated microRNA are included in the *Online Supplementary Table S1.*

Table 1. Comparison of microRNA expression (ΔΔCt method) by real-time PCR between mesenchymal stromal cells (MSC) from healthy donors (HD) and MDS patients.

miRNA	MSC-HD median (range)	MSC-MDS median (range)	*P*
155	0.81 (0.40-2.04)	0.33 (0.07-42.99)	0.015
181a	1.98 (1.19-4.17)	1.0 (0.15-3.25)	0.05
222	50.11 (38-90)	35.26 (3.92-133.58)	0.082
150	0.002 (0.0006-0.13)	0.005 (0.001-0.1)	0.19
146a	0.13 (0.03-2.81)	0.08 (0.005-9.95)	0.408
125a-5p	1.20 (0.59-1.40)	1.06 (.42-11.94)	0.462
129-3p	1.97 (0.37-7.46)	1.93 (0.68-6.89)	0.606
153	1.29 (0.11-2.76)	0.44 (0.00-7.97)	0.606
34c-5p	1.07 (0.37-2.03)	0.90 (0.14-16.57)	0.897
26b	1.02 (0.55-1.32)	0.91 (0.33-5.89)	0.806

Table 2. Comparison of gene expression of selected target genes of down-regulated microRNA between mesenchymal stromal cells (MSC) from MDS patients (n=14) and healthy donors (HD; n=5).

Target gene	MSC-HD median (range)	MSC-MDS median (range)	*P*
SDF1	0.86 (0.53-2.66)	0.49 (0.05-1.22)	0.026
TP53	0.90 (0.60-2.61)	1.31 (0.28-4.26)	0.414
PTEN	0.89 (0.21-14.34)	1.16 (0.04-14.99)	0.733
MYC	1.74 (0.09-2.62)	1.28 (0.71-4.41)	0.882

genesis, small single- stranded RNAs that function as guide molecules in post-transcriptional gene regulation, covering a broad spectrum of processes, including apoptosis and hematopoiesis.[19] Very few studies have evaluated *DICER1* or *DROSHA* activity in MSC and they focused on their role in differentiation of MSCs.[20,21] Thus, to gain insight into the biological effects of low *DICER1* and *DROSHA* expression in MSC-MDS, we further analyzed mature microRNA expression profile, showing a trend towards global downexpression in MDS-MSC when compared with normal MSC. These results could be associated with the inherent biological diversity among MDS subtypes. Among the recruited validated target genes of the strongest down-regulated miRNAs we found a strikingly high representation of the *DICER1* gene. All these features could also suggest the action of a compensatory mechanism to improve miRNA biogenesis. One additional explanation could be alternative (non-canonical) *DICER* and *DROSHA*-independent mechanisms of miRNA biogenesis that have been recently described;[22-24] however, these were not evaluated in the present study. Together, all these findings (*DICER*, *DROSHA* and microRNA disregulation) suggest that the canonical machinery regulating microRNA biogenesis in MSC from MDS patients is impaired.

We wanted to evaluate the potential role of these deregulated microRNA in the MDS pathology. We were able to observe that 3 out of 10 miRNAs (miRNA-155, miRNA-181a and miRNA-222) showed lower gene expression in MSC from MDS patients when compared with those from healthy donors. Of note, the 3 microRNA previously referred to have relevant roles in normal and malignant hematopoietic processes.[19,25,26] The potential effects of these microRNA deregulation on hematopoietic stem cells remain unclear, but it has recently been shown that MSC may secrete microRNA as well as other nucleic acids and proteins, into microvesicles, suggesting a novel mechanism of intercelular communication and regulation.[27,28]

It must also be stressed that *DICER1* and *DROSHA* are target genes of these three miRNAs, so it could be hypothesized that complex mechanisms involving *DICER1-DROSHA*-microRNAs could be observed in MSC-MDS as compensatory mechanisms. Furthermore, many target genes of these miRNAs are involved in molecular pathways related to hematopoiesis.[19] In this context *MYC*, *PTEN*, *SDF1* and *TP53* are target genes of down-regulated miRNAs (miR-155 and miR-181a) which are involved in the control and regulation of hematopoiesis.[15-18] *SDF1*, that is down-expressed in MSC from MDS patients, is a molecule involved in migration and attachment of HSC to its niche. It has been proposed that the *SDF1-CXCR4* axis is impaired in MDS patients.[17] The rationale for this gene downexpression associated with a global down-regulated microRNA expression remains elusive. However, it is important to note that micro-RNAs can act not only as repressors but also as enhancers of gene expression,[29] suggesting that the regulatory mechanisms are very complex.

In summary, we describe for the first time that MSC from MDS patients show low gene and protein expression of *DICER1* and *DROSHA* which are involved in the microRNA biogenesis, as well as their target gene SBDS, confirming recent findings in a murine model. Furthermore, these cells showed several deregulated microRNAs, mostly down-regulated. Together, these findings suggest that MSC might be involved in MDS pathogenesis.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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