



Despite differential gene expression profiles pediatric MDS derived mesenchymal stromal cells display functionality *in vitro* ☆

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Abstract Pediatric myelodysplastic syndrome (MDS) is a heterogeneous disease covering a spectrum ranging from aplasia (RCC) to myeloproliferation (RAEB(t)). In adult-type MDS there is increasing evidence for abnormal function of the bone-marrow microenvironment. Here, we extensively studied the mesenchymal stromal cells (MSCs) derived from children with MDS. MSCs were expanded from the bone-marrow of 17 MDS patients (RCC: n = 10 and advanced MDS: n = 7) and pediatric controls (n = 10). No differences were observed with respect to phenotype, differentiation capacity, immunomodulatory capacity or hematopoietic support. mRNA expression analysis by Deep-SAGE revealed increased *IL-6* expression in RCC- and RAEB(t)-MDS. RCC-MDS MSC expressed increased levels of *DKK3*, a protein associated with decreased apoptosis. RAEB(t)-MDS revealed increased *CRLF1* and decreased *DAPK1* expressions. This pattern has been associated with transformation in hematopoietic malignancies. Genes reported to be differentially expressed in adult MDS-MSC did not differ between MSC of pediatric MDS and controls.

An altered mRNA expression profile, associated with cell survival and malignant transformation, of MSC derived from children with MDS strengthens the hypothesis that the micro-environment is of importance in this disease. Our data support the understanding that pediatric and adult MDS are two different diseases. Further evaluation of the pathways involved might reveal additional therapy targets.

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Introduction

Pediatric myelodysplastic syndrome (MDS) represents a range of disorders characterized by dysplastic morphology comprising in total less than 5% of pediatric hematological malignancies (Hasle et al., 2004). The spectrum of MDS ranges from refractory cytopenia of childhood (RCC) to advanced MDS with excess of blasts (RAEB) with increasing risk of leukemic transformation (Hasle et al., 2003). Survival has increased from 30 to 60% since hematopoietic stem cell transplantation (HSCT) is applied (Strahm et al., 2011; Sasaki et al., 2001; Woods et al., 2002). The pathophysiology of MDS is not fully elucidated. However, genetic predisposition, acquired cytogenetic abnormalities and abnormal immune responses have been linked to MDS (Strahm et al., 2011; Hasle and Niemeyer, 2011). These aspects do not explain the entire range of disease in pediatric or adult MDS. Recently, it has been suggested in adult MDS that impaired interaction between hematopoietic precursor cells and their bone-marrow microenvironment might contribute to the disease (Zhang et al., 2012). In children, no conclusive data is yet available.

Mesenchymal stromal cells (MSCs) have been identified as supporting cells of hematopoietic stem cells (HSC) *in vivo* and *in vitro* (Morikawa et al., 2009; Mendez-Ferrer et al., 2010; Sugiyama et al., 2006) and linked to disease, as aberrant MSC function was shown to contribute to the pathophysiology of malignant disorders in murine models (Raaijmakers et al., 2010; Schepers et al., 2013). Characteristics of MSCs from adult MDS patients have been extensively studied focusing on cytogenetic and molecular abnormalities (Blau et al., 2011; Lopez-Villar et al., 2009; Flores-Figueroa et al., 2008) as well as gene and protein expressions (Flores-Figueroa et al., 2008; Marcondes et al., 2008; Santamaria et al., 2012). In addition, abnormal immunomodulation (Marcondes et al., 2008; Wang et al., 2013; Zhao et al., 2012a) as well as decreased hematopoietic support (Zhao et al., 2012a; Ferrer et al., 2013) by MSCs has been reported in MDS. However, these data remain conflicting with other studies reporting no abnormalities in stromal function (Flores-Figueroa et al., 2008; Klaus et al., 2010; Alvi et al., 2001). Differences in results may be explained not only by a variety of MSC expansion protocols and experimental set-up, but also by the heterogeneity of the disease (Aizawa et al., 1999). Studies reporting on (cyto)genetics and function of MDS-MSC have been summarized in Supplementary Tables S1 and S2.

Pediatric MDS is a very rare disease and publications on the role of stroma in the ontogeny and maintenance of pediatric MDS are limited to a case report on aberrant hematopoietic support by MSC derived from an MDS patient with trisomy 8 (Narendran et al., 2004), a study using stroma cells of 7 MDS patients (Borojevic et al., 2004), and a gene-expression analysis of the stromal compartment by the same research group (Roela et al., 2007). Nevertheless these scarce reports suggest an aberrant support of hematopoiesis associated with an altered gene expression profile of MSCs.

In the present study we compared MSCs derived from children with RCC and RAEB(t)/MDS-AML to MSCs expanded from age-matched healthy controls. Biological characteristics, e.g., differentiation capacity and phenotype were analyzed. MSC function *in vitro* was evaluated by

immunomodulatory and hematopoietic assays. In addition, genome wide gene-expression profiles were studied using Deep-SAGE sequencing.

Materials and methods

Patients and MSC expansion

Children referred to our center for HSCT were included in this study according to a protocol (P08.001) approved by the Institutional Review Boards on Medical Ethics. Next to the bone-marrow of 10 healthy controls (HC, median age 7.4, range 1.1–16.4 years) being HSCT donors, bone-marrow of 17 MDS patients (10 RCC, 2 RAEB, 4 RAEBt, 1 MDR-AML) was collected at diagnosis and prior to treatment initiation. The WHO classification adapted for children was used for the classification of patients. (2) MSCs from children with RAEB, RAEBt and MDR-AML were grouped as advanced MDS to enable the comparison between advanced and RCC-MDS. In addition, bone-marrow after HSCT was collected from 9 children (4 RCC, 1 RAEB, 1 RAEBt, 3 MDR-AML) including 6 paired samples (Table 1).

MSCs were expanded and characterized as previously described (Calkoen et al., 2013). Briefly, bone-marrow mononuclear cells (MNCs) obtained after Ficoll separation were cultured in DMEM (Invitrogen, Paisley, UK) containing 100 U/mL penicillin/100 µg/mL streptomycin (P/S; Invitrogen) and 10% (v/v) fetal bovine serum (FBS; VWR International, Bridgeport, NJ, USA). Non-adherent cells were removed by refreshing the medium twice weekly. Upon reaching confluency MSCs were harvested, pooled and passaged for further expansion resulting in non-clonal MSCs. Phenotype (CD73, CD90, CD105 positive; CD3, CD31, CD34, CD45, CD86, HLA-DR negative) and differentiation capacity towards osteoblasts and adipocytes were investigated at passages 2–3 and 5–7, respectively. All but anti-CD105 (Ansell Corporation Bayport, MN) antibodies were derived from Becton Dickinson Biosciences (BD), San Diego, CA, USA. Culture supernatant was collected after reaching 80% confluency at passages 3–5 for measurement of cytokine production.

Cytogenetics

To exclude common chromosome abnormalities in MSCs and malignant cells, interphase fluorescence *in situ* hybridization (FISH) for chromosomes 7 and 8 was performed on MSCs from patients with known monosomy 7 or trisomy 8 using the following probes: Vysis LSI D7S486/CEP7 and LSI IGH/LSI MYC, CEP8 (Abbott Laboratories, Abbott Park, IL, USA) (Bronkhorst et al., 2011).

Chimerism analysis

Chimerism (donor or recipient origin) was studied by cytosine adenine (CA)-repeat analysis in MSCs cultured from the bone-marrow harvested after HSCT as previously described (Lankester et al., 2010).

Table 1 Characteristics of patients included in the study.

UPN	Sex	Age (yrs) at HSCT	Diagnosis	Donor type	Donor Source	Conditioning	Remark MSC	Cytogenetics in hematopoietic cells	Pre-HSCT MSC	Post-HSCT MSC
MSC-MDS001	M	8.9	RAEBt	MUD	BM	Bu, Cy, Mel, rATG		–	Yes	Yes + 7 months
MSC-MDS002	F	13.3	RAEB	MUD	PBSC	Bu, Cy, Mel, rATG		Trisomy 1, monosomy 7, trisomy 8	Yes	Yes + 4 months
MSC-MDS003	F	5.4	RCC	IRD	BM	Bu, Cy, Mel	Only post	Monosomy 7		Yes + 2 months
MSC-MDS005	M	6.1	RCC	ORD	PBSC/MSC	1st: Flu, Thio, rATG, 2nd: Treo, Flu, Campath		–	Yes	Yes + 2 months
MSC-MDS008	F	14.2	MDR-AML	IRD	BM	Bu, Cy, Mel	Only post	–		Yes + 2 months
MSC-MDS009	F	15.8	MDR-AML	ORD	PBSC/MSC	Thio, Treo Flu, rATG	Only post			Yes + 7 months
MSC-MDS011	F	10.2	RCC	MUD	BM	Flu Thio Campath		–	Yes	Yes + 3 months
MSC-MDS015	F	9.3	RCC	MUD	BM	Bu, Flu, rATG		Monosomy 7	Yes	Yes + 2 months
MSC-MDS017	M	14.0	RCC	MUD	BM	Bu, Cy, Mel, rATG		–	Yes	
MSC-MDS018	M	14.6	RAEBt	MUD	BM	Bu, Cy, Mel, rATG		–	Yes	
MSC-MDS019	F	4.8	RCC	MUD	BM	Bu, Cy, Mel, Campath		Monosomy 7	Yes	
MSC-MDS020	M	13.3	RAEB	MUD	BM	Bu, Cy, Mel, Campath		Monosomy 7	Yes	
MSC-MDS021	M	13.3	RAEBt	IRD	BM	Bu, Cy, Mel, rATG		Trisomy 8	Yes	
MSC-MDS022	F	4.1	RCC	MUD	CB	Bu, Cy, Mel, rATG		Monosomy 7	Yes	
MSC-MDS023	F	1.2	RAEBt	MUD	BM	Bu, Mel, ARA-C, rATG		Monosomy 7	Yes	
MSC-MDS024	M	4.1	MDR-AML	IRD	BM	1st: Flu Thio Campath, 2nd: Flu Thio treo rATG, 3rd none		–	Yes	Yes + 3 months
MSC-MDS026	M	17.8	RCC	MUD	BM	Flu, Thio, Campath		–	Yes	
MSC-MDS027	M		RCC	n.a.			No HSCT	–	Yes	
MSC-MDS028	F		RCC	n.a.			No HSCT	–	Yes	
MSC-MDS029	F	17.6	RCC	MUD	BM	Flu, Thio, Campath		–	Yes	

RCC: refractory cytopenia of childhood; RAEB: refractory anemia with excess of blasts; RAEBt: RAEB in transformation; MDR-AML: myelodysplasia related acute myeloid leukemia; HSCT: hematopoietic stem cell transplantation; MUD: matched unrelated donor; IRD: identical related donor; ORD: other related donor; BM: bone marrow; PBSC: peripheral blood stem cells; MSCs: mesenchymal stromal cells; CB: cord blood; Bu: busulphan; Cy: cyclofosamide; Mel: melphalan; rATG: rabbit anti-thymocyte globulin; Flu: fludarabine; Thio: thiopeta; Treo: treosulphan; ARA-C: cytosine arabinoside; n.a.: not applicable.

Immunomodulatory assays

The effect of MSCs (30 Gy irradiated) on proliferation of peripheral blood (PB) MNCs obtained from adult blood bank donors (100,000 cells/well) after stimulation with phytohemagglutinin (PHA 2 $\mu\text{g}/\text{mL}$) was analyzed at MSC:PBMC ratios of 1:5 and 1:40. MSCs and PB-MNCs were co-cultured in RPMI P/S, 10% (v/v) fetal calf serum (FCS) for 5 days with the addition of ^3H -thymidine (1 $\mu\text{Ci}/\text{well}$; Perkin Elmer, Wellesley, MA, USA) for the last 16 h to measure proliferation using a β -counter (Perkin Elmer). Experiments were performed in triplicate.

To evaluate the effect of MSCs on antigen presenting cells, monocytes were isolated from PB using positive CD14 selection (Miltenyi, Bergisch Gladbach, Germany) and cultured with IL-4 (40 ng/mL) and GM-CSF (800 IU/mL) (both from Tebu-Bio, Le Perray en Yvelines, France) for 5 days to differentiate towards immature dendritic cells (DCs). Cells were harvested or cultured for 2 additional days with IL-4, GM-CSF, IFN- γ (500 U/mL, Boehringer, Mannheim, Germany) and CD40-ligand (0.25 $\mu\text{g}/\text{mL}$ Beckman-Coulter, Marseille, France) to generate mature DCs. Cells were phenotyped by flow cytometry for the expression of CD14 and CD1a (BD) on day 0, day 5 and day 7 after co-culturing of monocytes and MSCs at MSC:monocyte ratios of 1:5, 1:40 or 1:100 or after culturing monocytes without MSCs.

Hematopoietic support

Short-term co-culture assays with MSCs and hematopoietic progenitor cells (HPCs) were performed to determine the supportive capacity of MSCs for HPC maintenance and differentiation. Therefore, HPCs were isolated from the remaining material of G-CSF mobilized stem cell grafts from healthy transplant donors using CD34 positive selection (Miltenyi). Selected cells expressed >90% CD34 after purification. Short-term cultures of 500 CD34 selected cells/well without or with MSCs (CD34:MSC ratios of 1:2 and 1:20) were performed in Stemspan medium (H3000, StemCell Technologies, Vancouver, Canada) with addition of 1% P/S, stem cell factor (SCF, 100 ng/mL, StemCell Technologies) and Flt3-ligand (Flt3-L, 100 ng/mL, StemCell Technologies), because SCF and Flt3-L are not produced by MSCs. Cultures were initiated with 10×10^3 CD34+ cells at a CD34:MSC ratio of 1:5 for flow cytometry analysis. Half of the culture medium was refreshed with the addition of growth factors on days 4, 7 and 11. Proliferation (day 7) and differentiation (days 7 and 14) were assessed using ^3H -thymidine during the last 16 h or flow cytometry, respectively. Antibodies used for flow cytometry were anti-CD34-PE, anti-CD45-FITC, anti-CD38-Perpcp5.5, anti-CD45-Perpcp5.5, anti-CD14-FITC, anti-CD33-APC, anti-GPA-PE (glycophorin A) and anti-CD13-PE (all antibodies from BD).

In long-term cultures, CD34 selected cells (50,000 cells) were cultured on a confluent MSC layer for 3–5 weeks in the absence of growth factors. Cells were harvested and counted using trypan blue as viability stain.

To determine the functional impact of HPC expansion and differentiation after short-term culture, non-adherent cells were harvested after 7 days of culturing CD34+ cells in the absence or presence of MSCs (CD34:MSC ratio of 1:5),

transferred (1000 cells/dish) to methylcellulose containing essential growth factors, *i.e.*, SCF, GM-CSF, IL-3, and erythropoietin (EPO) (H4434 StemCell Technologies), and cultured for 14 days (colony-forming unit assay; CFU-assay). Colonies were scored by two independent observers according to standard guidelines for the definition of CFU-GEMM, BFU-e, CFU-GM, CFU-G and CFU-M. Results are depicted as the average of duplicate wells. To determine a more direct effect of MSCs on HPCs in CFU-assays, MSCs (30,000 or 150,000 per dish) were added to freshly purified HPCs (500 cells/dish) in methylcellulose containing SCF, GM-CSF, IL-3 and EPO (H4434 StemCell Technologies). The direct effect of MSCs on colony formation was also assessed in methylcellulose containing EPO (H4330 StemCell Technologies) only. Cells were harvested and phenotyped after scoring of colonies in the CFU-assay.

Cytokine expression

IL-6 quantification in MSC culture supernatants was performed by ELISA (Sanquin, Amsterdam, the Netherlands) according to the manufacturer's instructions.

Gene expression

Total RNA was isolated at passages 2–3 using a Qiagen RNeasy Minikit (Qiagen, Hilden, Germany). mRNA was profiled using Deep-SAGE sequencing using Illumina technology (Mastrokolias *et al.*, 2012). CATG was added to the 5' end of the 17 base pair sequences obtained. Data were mapped against the UCSC hg19 reference genome using Bowtie for Illumina (version 1.1.2) without the permission of one mismatch and suppression of reads if more than one best match existed. Tags aligned to the same gene were summed for further analysis. Gene information was added to the sequences with the biomaRt package in R (version 2.16.0). The expression data will be published online in the Gene Expression Omnibus (GEO).

Expression of genes of interest was validated using independent biological samples by RT-PCR after generation of cDNA (cDNA synthesis kit, Roche, Basel, Switzerland) using the listed primers (Table S3), as previously described (Mastrokolias *et al.*, 2012). Expression levels were calculated relative to expression of the housekeeping genes *GAPDH* and *HPRT1*.

Statistical analysis

Graphpad 6 (Prism, La Jolla, CA) was used for data-analysis. Mann–Whitney and Wilcoxon matched-pairs signed rank tests were performed to compare different groups. Differential gene expression analysis was performed in R (version 2.15.0), using the EdgeR (version 3.2.4) and Limma (version 3.16.7) data analysis packages (Robinson *et al.*, 2010; Anon., 2012; Smyth *et al.*, 2005). Correction for multiple testing was performed according to Hochberg and Benjamini (1990). Adjusted *p*-values < 0.05 were considered statistically significant.

Results

Expansion and characterization of MSC

MSCs were successfully cultured from the bone-marrow of all patients and controls. Expanded MSCs expressed CD73, CD90 and CD105, whereas the cells did not express lineage

markers (Supplementary Fig. S1A). MSCs differentiated towards adipocytes and osteoblasts when cultured in culture media supporting these distinct directions of differentiation (Figs. S1B–E). In one *de novo* RCC patient (UPN: MDS026) no osteoblast differentiation was established. Post-HSCT derived MSCs were of complete patient origin in all children that were analyzed (n = 6). Monosomy 7 was not detected in

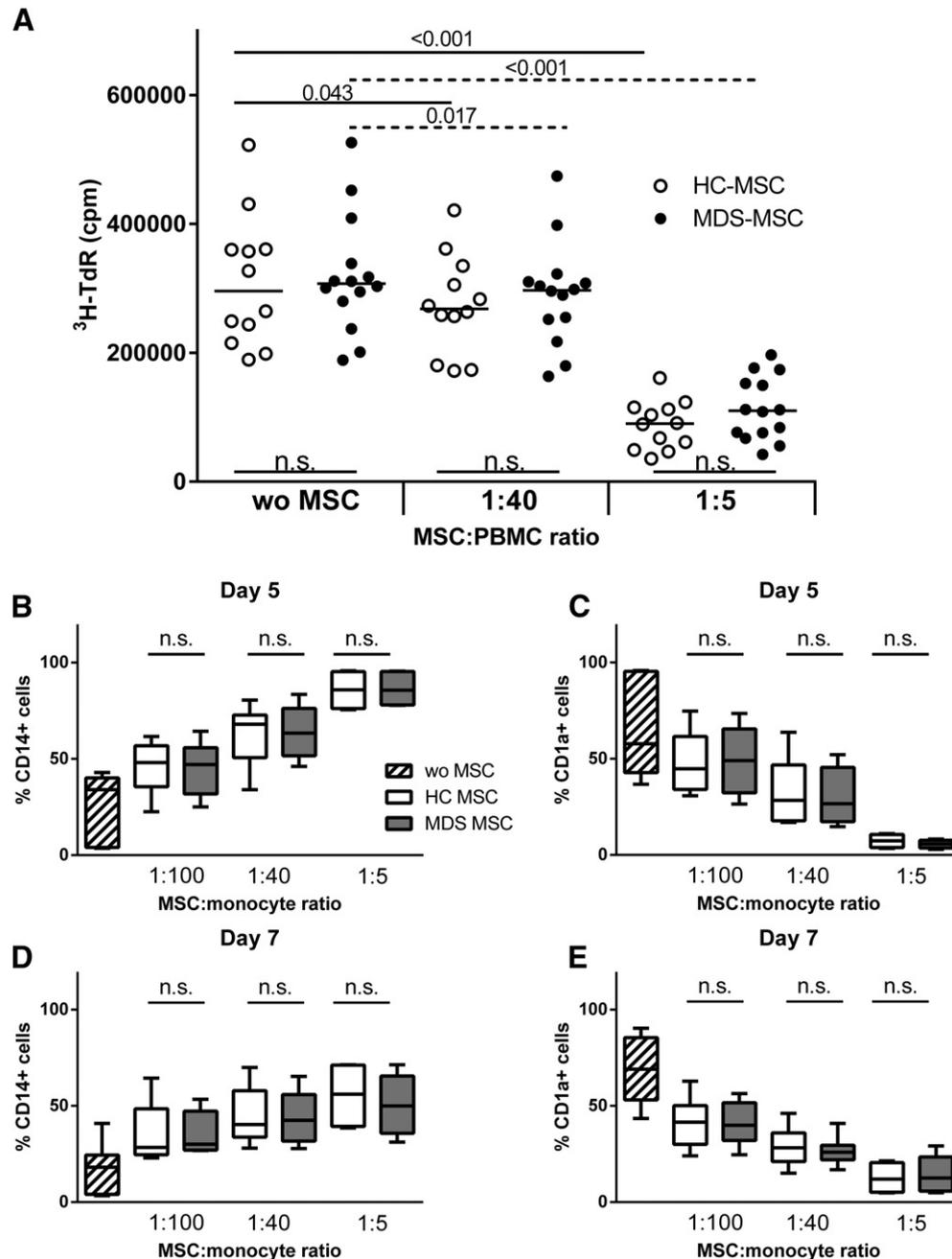


Figure 1 Immunomodulation by MDS-MSC. A. Both healthy control (HC, n = 6) and MDS-MSCs (n = 5 of which RAEB/RAEBt n = 3 and RCC n = 2) significantly suppressed PHA-induced PBMC (n = 4 healthy adults) proliferation at MSC:PBMC ratios of 1:40 and 1:5. No significant differences were observed between MDS patients (black circles) and controls (white circles). wo MSC: without MSC. B–E. MSCs suppressed the differentiation of monocytes (CD14⁺ cells) towards dendritic cells (CD1a⁺ cells) in a dose-dependent manner (hatched boxes: no MSC added). No significant differences were observed between HC (white boxes) and MDS-MSC (black boxes). Boxes represent median and 25–75 percentiles and the whiskers the minimum and maximum values. *p*-Values were calculated using Wilcoxon matched-pairs signed rank tests (in A for comparison of different MSC ratios) and Mann–Whitney tests (in A–E for comparison between HC and MDS). n.s.: not significant.

MSCs from any bone marrow harvested prior to HSCT from children with monosomy 7 ($n = 6$). The MSC lines generated from the two patients with trisomy 8 in the hematopoietic cell compartment tested negatively for trisomy 8.

Immunomodulation

MSCs down modulate functions of various cell types involved in innate and adaptive immunity (Le Blanc and Mougiakakos, 2012). The effect of MSCs of pediatric MDS patients and healthy children on T-cell proliferation and monocyte differentiation was investigated. MSCs suppressed the PHA-induced PB MNC proliferation in a dose-dependent manner. No differences in suppressive capacity were observed between MDS-MSCs and healthy control (HC-)MSCs (Fig. 1A).

To investigate the suppressive effect of MSCs on DC maturation, MSC-monocyte co-cultures were performed. The purified monocyte fraction was $>95\%$ CD14⁺ and $<1\%$

CD1a⁺ at the start of MSC-monocyte co-culture. After 5 days of culture with GM-CSF and IL-4, the monocytes lost CD14 expression and gained CD1a, characteristic for DCs. This process further progressed from day 5 to 7 during maturation of DCs in the presence of GM-CSF, IL-4, IFN- γ and CD40-ligand. MSCs of controls and MDS patients showed inhibition of the differentiation at days 5 and 7 at various MSC:monocyte ratios (Figs. 1B–E). No differences between both groups were observed. RAEB(t)-MSCs and RCC-MSCs showed comparable suppressive effects in both assays (data not shown).

Expansion of hematopoietic progenitor cells

As part of the stromal bone-marrow compartment, MSCs play an important role in the regulation of hematopoiesis through interaction with HPCs (Mendez-Ferrer et al., 2010). The effect of MSCs of pediatric MDS patients and healthy children

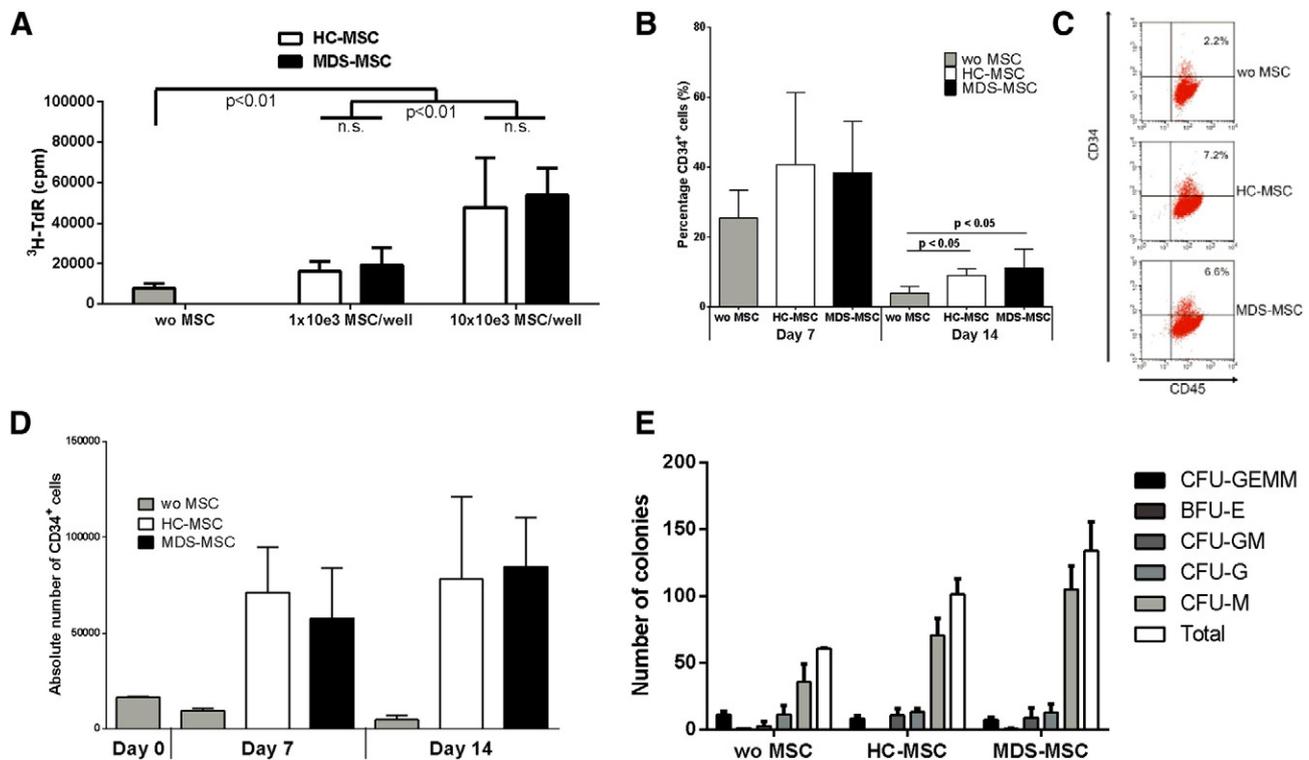


Figure 2 MDS-MSCs support maintenance and differentiation of hematopoietic progenitor cells (HPCs). A. CD34⁺ cells (500 cells) of HSCT donors were cultured in the absence (wo MSC) or presence of MSCs (CD34:MSC of ratios 1:2 and 1:20) obtained from MDS patients or healthy controls (HC) in the presence of SCF and Flt3-L. Proliferation of HPCs at day 7 was assessed by ³H-thymidine incorporation. B–C. CD34⁺ expression declined overtime, but MSCs supported the expansion of HPCs (CD34⁺ cells) and a higher percentage of CD34⁺ cells were retained in comparison with cultures in the absence of MSCs. Results shown are from cultures at a CD34⁺:MSC ratio of 1:5 starting with 10×10^3 HPCs. Flow cytometry data presented in C are obtained after 14 days of culture and show plots of CD34 versus CD45 expression and the percentage of CD34⁺ cells within the CD45⁺ cell population. D. Whereas the absolute number of HPCs decreased significantly in the absence of MSCs, the absolute number of HPCs increased in co-cultures with MSCs (CD34⁺:MSC ratio of 1:5). Data depicted in A, B and D represent at least 2 independent experiments of 5 HC-MSCs and 8 MDS-MSCs (gray bars: without (wo) MSCs, white bars: healthy control (HC)-MSCs, black bars: MDS-MSCs). E. Non-adherent cells harvested at day 7 were transferred to methylcellulose to test their capability of colony formation. Graphs represent the total number of CFU and indicated CFU types in the CFU-assay resulting from the investigation of 4 MDS-MSCs and 3 HC-MSCs present during the initial HPC and MSC co-culture. wo MSC: without MSCs. Bars depict the mean with standard deviation. *p*-Values were calculated using Mann–Whitney tests. n.s.: not significant.

on expansion and differentiation of CD34⁺ HPCs was investigated in various *in vitro* culture systems.

HPC (CD34⁺ cells) proliferation was enhanced in the presence of MSCs in a dose-dependent manner after stimulation with SCF and Flt3-L for 7 days (Fig. 2A). The percentage of cells expressing CD34 declined over time, but the level of this decline is significantly less in cultures containing MSCs (Figs. 2B–C). In co-cultures of HPCs with MSCs, CD38 expression, associated not only with activation but also with loss of stemcellness (Calloni et al., 2013; Chillemi et al., 2013), was significantly increased at day 14 on both CD34⁺ and CD34[−] cells (CD34⁺ cells, mean and SD: without MSCs: 18.8 ± 1.5%; plus HC-MSCs: 46.8 ± 8.9%; plus MDS-MSCs: 52.0 ± 5.0%. CD34[−] cells: without MSCs 25.9 ± 2.9%; plus HC-MSCs: 54.9 ± 9.4%; plus MDS-MSCs: 61.5 ± 5.3%). In cultures with MDS-MSCs, differentiation towards myeloid-lineage cells was not enhanced in comparison with cultures with HC-MSCs or without MSCs (CD14⁺ cells as a percentage of CD45⁺ cells, mean and SD: without MSC: 16.2 ± 2.0%; plus HC-MSCs 16.0 ± 2.5%; plus MDS-MSCs: 19.0 ± 2.2%).

The absolute number of CD34⁺ cells decreased significantly when HPCs were cultured in the absence of MSCs. In contrast, in the presence of MSCs, the HPC (CD34⁺) population expanded from day 0 to day 7. From day 7 to day 14 the CD34⁺ numbers remained unchanged compared to day 7 (Fig. 2D).

Non-adherent cells (1000 cells), harvested from HPC cultures with or without MSCs at day 7, were transferred to methylcellulose for CFU-C analysis. Cells that have previously been co-cultured with MSCs gave rise to higher numbers of CFU-C, compared to HSCs that have been cultured in the absence of MSCs (Fig. 2E). This is in accordance with the increased CD34⁺ numbers that were observed (Fig. 2D). No differences were found in the types of colonies that were formed and this was independent of the presence or absence of MSCs. Overall, the impact of MSCs in these various assays of HPC function was similar for MDS patients and healthy controls, as well as for RAEB(t) *versus* RCC patients (data not shown).

Maintenance of hematopoietic progenitor cells

To exclude the influence of exogenous growth factors on HPC expansion and the capacity to mount colonies, 50 × 10³ CD34⁺ cells were seeded on confluent MSC layers and cultured for 3–5 weeks without the addition of growth factors. When cultured in the absence of MSCs, all CD34⁺

cells died. In contrast, the CD34⁺ cells were maintained when cultured in the presence of MSCs. The number of viable cells harvested following 3–5 weeks of culture did not differ between MDS-MSCs (n = 4) and HC-MSCs (n = 2) (Fig. S2A). Non-adherent cells harvested after 3 weeks of culture in the presence of HC-MSCs or MDS-MSCs formed similar numbers of colonies in CFU-assays (range 12–27 vs 8–32, respectively) (Fig. S2B).

Support of colony formation

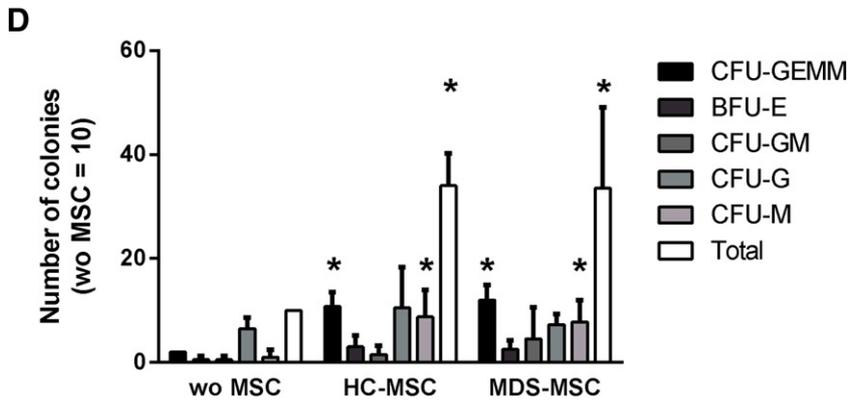
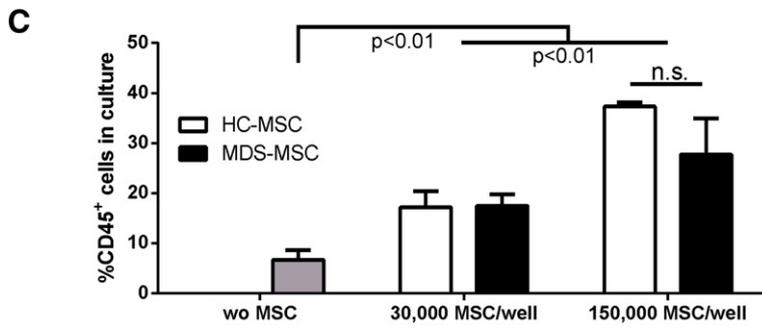
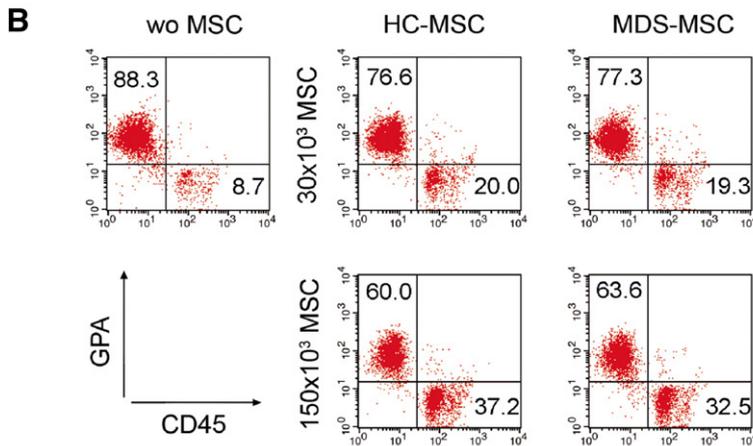
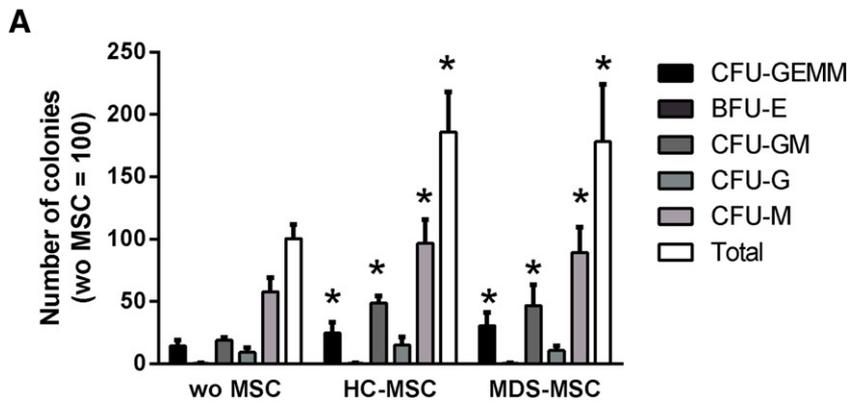
To study the direct influence of MSCs on colony formation by freshly isolated HPCs, MSCs (30 × 10³ or 150 × 10³ cells/well) were added to purified HPC (500 cells/well) in methylcellulose containing growth factors, *i.e.*, SCF, GM-CSF, IL-3, and EPO. The total number of colonies at day 14 was increased by the addition of MSCs (*p* = 0.01). A significant increase was seen in CFU-GEMM, CFU-GM and CFU-M (Fig. 3A). The proportion of CFU-GM colonies was increased when cells were cultured in the presence of MSCs compared to CFU-C assays in the absence of MSCs. In accordance with this, the percentage of CD45⁺GPA[−] myeloid cells were increased in the non-adherent cell population (colonies) harvested at day 14 from cultures containing MSCs (Figs. 3B–C). No differences in the supportive effect on colony formation and HPC differentiation were observed between MDS-MSCs (n = 6) and HC-MSCs (n = 4). When HPCs were cultured in the presence of HC-MSCs (n = 3) or MDS-MSCs (n = 3) in methylcellulose with erythropoietin and without GM-CSF, SCF and IL-3 the number and size of colonies were significantly increased in comparison with cultures without MSCs (Fig. 3D). In conclusion, HC-MSCs and MDS-MSCs have similar effects on colony formation *in vitro*.

Gene and protein expressions

In functional assays no evidence was obtained for a disturbed MSC function in children with MDS. However, these functional studies are limited and the results do not formally exclude the possible existence of biologically relevant differences between MDS-MSC and HC-MSCs. To further investigate this Deep-SAGE was performed on total RNA identifying the expression of all mRNA from the 3′-end.

MSCs derived from RCC (n = 4), RAEB(t) (n = 4) and healthy controls (HC, n = 8) were analyzed. A median of 15.9 × 10⁶ reads (range 9.5 × 10⁶–30.6 × 10⁶) was obtained. Between 59.3% and 68.4% (median 65.6%) of reads were

Figure 3 MSCs of MDS patients support colony formation in CFU-assays. A–C. MSCs (30,000 cells, or 150,000 cells when indicated) of healthy controls (HC-MSC), of MDS patients (MDS-MSC) or no MSCs (wo MSC) were added to freshly isolated HPCs (CD34⁺ cells, 500 cells) of HSCT donors at initiation of colony forming unit assays (CFU-assay). Cultures contain exogenous SCF, GM-CSF, IL-3 and EPO. Addition of MSCs supports the increase of the number of colonies (MDS n = 6, HC n = 4). To compare data from different experiments, the number of colonies in cultures without MSCs (range: 83–105) was set at 100. Colonies were scored and harvested for phenotyping at day 14 after culture initiation. The percentage (B) and number (C) of CD45⁺GPA[−] cells are increased in CFU-assays in the presence of MSCs, irrespectively whether MSCs were derived from MDS patients or healthy controls (HC). D. MSCs from MDS patients (n = 3) and healthy controls (HC; n = 3), co-cultured with HPCs in the presence of EPO only, significantly increase the formation of CFU-GEMM and CFU-M, but not of the other colony types (read-out CFU-assay at day 14). wo MSC: without MSCs. Bars represent mean and standard deviation. *p*-Values were calculated using Mann–Whitney tests. *: statistically different (*p* < 0.05) from culture without (wo) MSCs. n.s.: not significant.



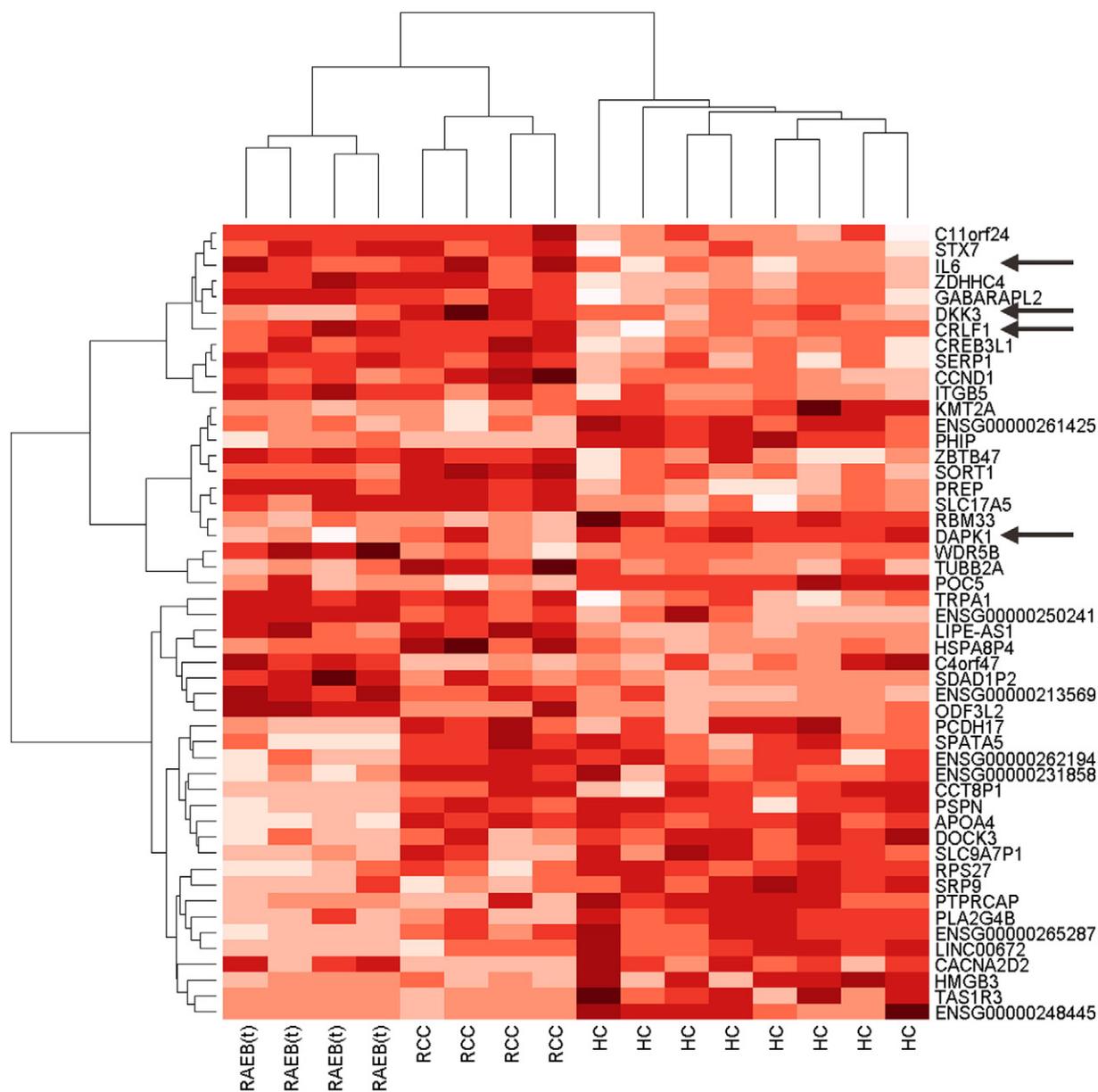


Figure 4 Heat map depicting clustering of MDS derived MSCs. Gene expression was analyzed using LIMMA software. MSCs of different groups (RAEB(t)-MDS, RCC-MDS and healthy controls (HC)) showed hierarchical clustering in a heat map of differentially expressed genes. Color intensity of the squares correlates with increased gene expression.

aligned to the genome using Bowtie; with a median of 55.4% (min 45.3%; max 57.6%) of the reads being mapped to an exon.

A heat map reflecting the top 50 differentially expressed genes demonstrates clustering of healthy control, RCC and RAEB MSC (Fig. 4). The gene expression profile of RCC clustered more towards healthy controls than RAEB(t). After correction for multiple testing, *IL6* and dickkopf 3 homologue (*DKK3*) were significantly expressed higher in RCC-MSCs compared to HC-MSCs ($p = 0.002$ and $p = 0.005$, respectively). Death-associated protein kinase 1 (*DAPK1*) expression was decreased ($p = 0.049$) in RAEB(t)-MSCs compared to HC-MSCs, whereas cytokine receptor-like factor 1 (*CRLF1*) and *IL6* expressions were increased ($p = 0.009$ and $p = 0.048$,

respectively). Differential expression of *IL6*, *DKK3*, *DAPK1* and *CRLF1* was confirmed by RT-PCR (Fig. 5A). In addition, the IL-6 concentration in culture supernatants of MSCs from the bone-marrow obtained at diagnosis was significantly increased in all MDS cases ($n = 10$) compared to supernatants of healthy control MSCs ($n = 8$) ($p < 0.001$; Fig. 5B).

Deep-SAGE and RT-PCR showed that IL-6 expression was elevated in MSCs of both RCC and RAEB(t) obtained prior to HSCT. Of note, after HSCT, *IL6* and *DAPK1* expression levels in MSCs were comparable to HC-MSCs (Fig. 5C). IL-6 concentration in MDS-MSCs culture supernatant was, although lower than in MSC samples generated from the bone-marrow taken pre-HSCT, still significantly higher than in supernatant of HC-MSCs (Fig. 5B). *DKK3* and *CRLF1*

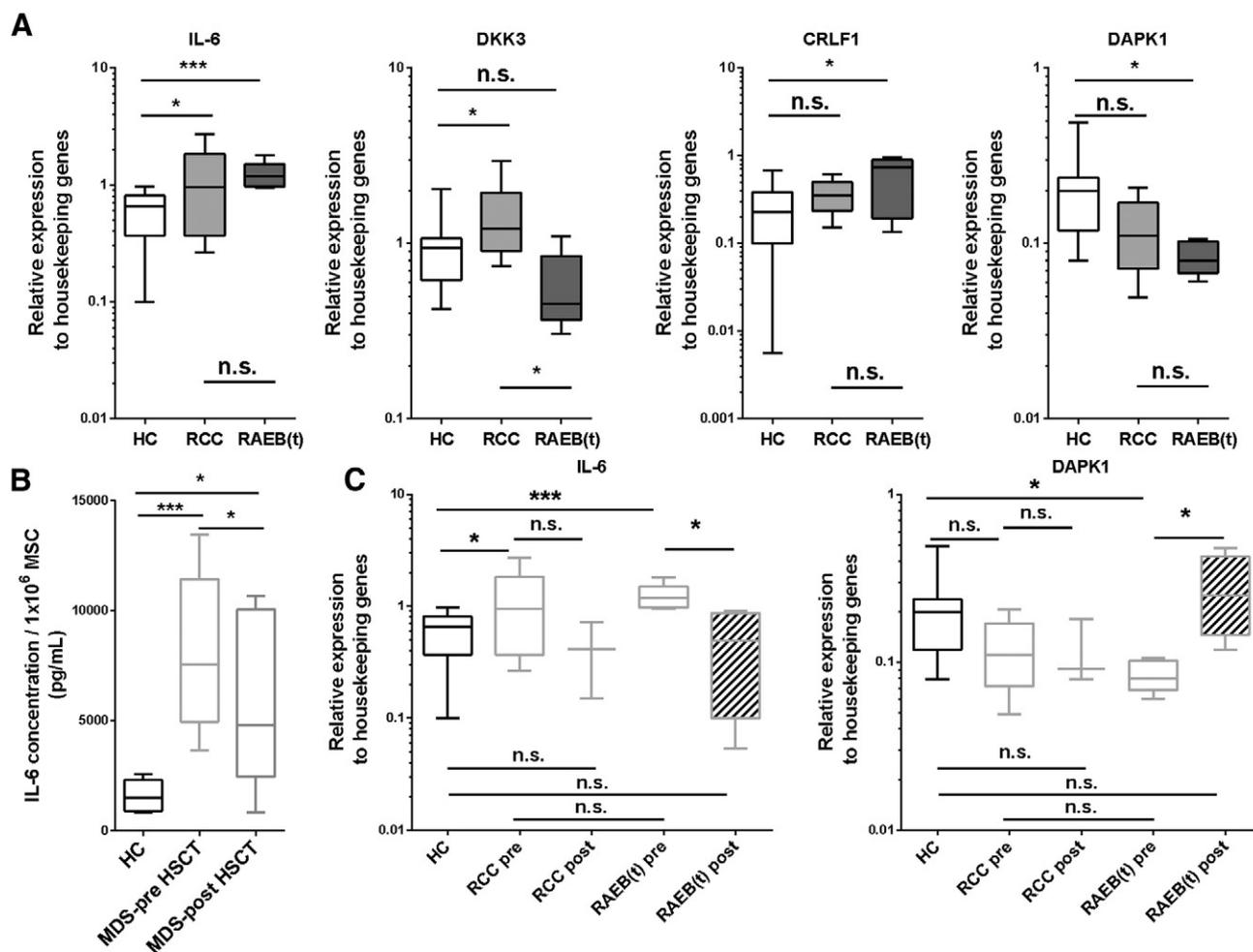


Figure 5 Differential mRNA expression by MSCs of MDS patients. **A:** Aberrant gene expression detected by Deep-SAGE was confirmed by RT-PCR. *IL-6* expression was increased in MSCs of both RCC and RAEB(t) MDS patients at diagnosis compared to MSCs of healthy controls (HC). In contrast, *DKK3* was specifically increased in RCC-MDS, whereas *CRLF1* and *DAPK1* were significantly altered in RAEB(t)-MDS derived MSCs. Data were normalized using GAPDH and HPRT1 as house-keeping genes (HC-MSC n = 10, RCC-MSC n = 7 and RAEB(t)-MSC n = 4). **B:** Increased *IL-6* gene expression in MSCs of MDS patients pre-HSCT (n = 10) compared to HC-MSCs (n = 8) was confirmed by ELISA quantifying IL-6 secreted by MSCs in culture supernatant. IL-6 concentration was still elevated in supernatant of MSCs generated from the bone-marrow obtained after HSCT, although there is a trend to normalization (n = 7). **C:** Gene expression analysis demonstrated a normalized mRNA expression in MDS-MSCs after HSCT compared to HC-MSCs for *IL-6* and *DAPK1*. RCC: refractory cytopenia; RAEB(t): refractory anemia with excess of blasts (in transformation); HC: healthy control; *DKK3*: dickkopf 3 homologue; *CRLF1*: cytokine receptor-like factor 1; *DAPK1*: death-associated protein kinase 1; HSCT: hematopoietic stem cell transplantation; boxes represent median and 25–75 percentiles with whiskers marking the range; Mann–Whitney statistical tests were performed to compare the different groups; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. n.s.: not significant.

expressions in MSCs expanded after HSCT from RCC and RAEB(t) patients, respectively, were not significantly altered compared to MDS-MSCs before HSCT or to HC-MSCs (data not shown).

Potential candidate genes based on their reported differential expression in adult MDS-MSCs, *i.e.*, *AURKA*, *AURKB*, *SCF*, *G-CSF* and *GM-CSF* (Santamaria et al., 2012; Ferrer et al., 2013; Zhao et al., 2012b; Oliveira et al., 2013), were specifically analyzed and no differences were observed comparing RCC-MSCs, RAEB(t)-MSCs and HC-MSCs.

Similarly, a comparable expression level of *CXCL12*, *Dicer1* and *Drosha* in pediatric MDS-MSCs versus HC-MSCs was confirmed by RT-PCR (data not shown).

Discussion

The spectrum of pediatric MDS ranges from aplasia to myeloproliferative disease. The pathophysiology of the disease has been attributed to different cytogenetic abnormalities (Gohring et al., 2010). Previous studies in mice and in human adults have linked the interaction of hematopoietic progenitors and the micro-environment to the progression of disease in several hematopoietic disorders (Zhang et al., 2012; Schepers et al., 2013). In adult MDS specific alterations in the MSCs have been reported as summarized in Table S1. Data on pediatric MDS are limited (Table S2). In this study, we compared the MSC characteristics and

function in children with different types of MDS with healthy controls. Differences were neither observed with respect to the differentiation capacity of MSCs, their immunomodulatory capacity using T-cell proliferation and monocyte differentiation to dendritic cells as read-out, nor regarding their impact on maintenance and differentiation of hematopoietic progenitor cells. In addition, cell viability in co-cultures was equally increased by both groups of MSCs, as assessed by trypan-blue staining (data not shown).

However, evaluation of total mRNA expression profiles demonstrated gene expression differences between MSCs derived from pediatric MDS patients and controls. Cytogenetic abnormalities present in the hematopoietic cells could not be detected in the stromal compartment, and, therefore, this cannot explain the differential gene expression. The partial normalization of *IL6* and *DAPK1* expressions in MSCs after HSCT in these patients demonstrates that the expression differences can be reversed. Of note, using chimerism analysis, the presence of donor MSCs in the expanded cells has been excluded.

Differential gene expression between pediatric MDS in general and healthy controls was most prominent for *IL6*. This gene has previously been reported to be over-expressed in adult MDS and in one child with MDS and a constitutional trisomy 8 (Zhao et al., 2012a; Narendran et al., 2004). In contrast, other studies in adults did not show differential *IL6* expression between MDS patients and healthy controls (Flores-Figueroa et al., 2002, 2008; Zhao et al., 2012a; Klaus et al., 2010). *IL6* has been described to increase myeloid differentiation via *STAT3* activation and support multiple myeloma cell growth and survival (Minami et al., 1996; Zhang et al., 2010; Cszasz et al., 2013; Gunn et al., 2006). *STAT3* up-regulation was not observed in this pediatric MDS cohort. In addition, IL-6 is one of the cytokines responsible for bone-remodeling in inflammatory and malignant diseases (Ara and Declerck, 2010; Dayer and Choy, 2010). Suppression of monocyte to dendritic cell differentiation is dependent on IL-6 (Melief et al., 2013). However, we did not observe a correlation between the degree of the suppressive effect of MSCs and the level of *IL-6* expression, suggesting that IL-6 is not the sole factor hampering this differentiation.

Besides *IL6* (RCC- and RAEB(t)-MDS), *DKK3* (RCC-MDS), *CRLF1* and *DAPK1* (RAEB(t)-MDS) were differentially expressed by MSCs of healthy controls versus MDS. *DKK3* and *CRLF1* have been associated with increased cell survival by suppressing apoptosis in MSCs and neuroblastoma cells, respectively (Song et al., 2006; Looyenga et al., 2013). Differential expression of these genes did not correlate with MSC expansion rates (data not shown). In addition, increased *CRLF1* in combination with IL-6 has been described in idiopathic pulmonary fibrosis causing inflammation, but suppression of fibrosis (Kass et al., 2012). *DAPK1* down-regulation, associated with malignant transformation, has been described in the hematopoietic cells in adult RAEB(t)-MDS potentially attributing to aberrant methylation (Raval et al., 2007; Qian et al., 2010; Wu et al., 2011; Claus et al., 2012; Karlic et al., 2013). We demonstrate a similar expression profile in our MSCs, with expression in RCC-MDS being similar to HC-MSCs, and down-regulation in RAEB(t)-MSCs. After successful HSCT, *DAPK1* expression was normalized. The allogeneic HSCT procedure leading to elimination of derailed cells and restoration of hematopoiesis through donor HPCs

might contribute to normalization of the stromal environment in the hematopoietic niche, including MSCs of recipient origin.

Analysis of total mRNA expression profiles not only revealed differences between RCC and RAEB(t)/MDR-AML in children, but also enabled us to specifically focus on genes previously reported to be differentially expressed in adult MDS. Genes of interest included micro-RNAs reported by Santamaria et al. (2012) as well as genes encoding cytokines, their receptors, chemokines and adhesion molecules. In contrast to what has been described for adult MDS, *AURKA*, *AURKB*, *SCF*, *G-CSF*, *GM-CSF*, *CXCL12*, *Dicer1* and *Drosha* were not differentially expressed in our cohort of pediatric MDS patients compared to healthy controls. Lack of differences in expression levels of *Dicer1*, *Drosha* and *CXCL12* was further confirmed by RT-PCR (data not shown). This supports the current understanding that pediatric and adult MDS are two different diseases as previous studies have highlighted the differences between adult and pediatric MDS, e.g., in response to treatment and rarity and prognostic value of (epi-)genetic mutations in the hematopoietic compartment (Hasle et al., 2004; Glaubach et al., 2014; Hirabayashi et al., 2012). Besides IL-6, genes included in the clustered analysis did not encode for molecules known to be involved in MSC signaling as reviewed by Le Blanc and Mougiakakos (2012).

Our MDS cohort is heterogeneous containing RCC as well as advanced MDS patients. Bone-marrow post HSCT was not available in all cases, because informed consent was limited to bone-marrow sampling on clinical indication namely relapse risk and non-engraftment. Correlation of mRNA expression in MSCs obtained at diagnosis with MSCs at MDS relapse after HSCT was not feasible due to low sample numbers in combination with limited numbers of relapse after HSCT.

Our findings demonstrate differences in mRNA expression between pediatric MDS and age-matched healthy control derived MSCs. This is in accordance with published data on MSCs derived from adults with MDS, however, as expected, not all abnormalities described in adults were present in pediatric MDS. In addition, different expression levels of specific genes were not associated with functional aberrations in assays pointing to immunomodulation and hematopoiesis, potentially caused by compensatory mechanisms or insufficient sensitivity of our tests. Growth differences between MSC precursors and use of non-clonal MSC populations may lead to loss of information and, thereby, to potential loss of differences between pediatric MDS and healthy control derived MSCs. Unfortunately, data on the interaction of MSCs with MDS patient derived hematopoietic stem cells was limited by the available material. However, preliminary data do not reveal differences in co-cultures of MDS-RAEB HPCs with MDS patient or healthy control derived MSCs.

Studying the pathogenesis of MDS has been complicated by the poor engraftment of human MDS HPCs in immunodeficient mice (Thanopoulou et al., 2004). However, co-transplantation of stromal cells and intramedullary transplantation of hematopoietic cells have led to increased engraftment (Kerbaui et al., 2004). Knockout models resulting in an MDS-like phenotype or the use of scaffolds with patient-derived MSC to resemble the human bone-marrow microenvironment might be instrumental in further exploring the potentially functional implications of these differences in future studies (Raaijmakers et al., 2010; Groen et al., 2012; Walkley et al., 2007).

In conclusion, our data show that the gene expression profile is different in MSC of children with MDS. It remains to be elucidated whether the abnormalities are a cause or a consequence of the disease. Normalization of the aberrant gene expression seen in patients derived MSC after allogeneic HSCT is an argument favoring the latter possibility. Induced abnormalities in the MSC by dysplastic cells might be targets to sustain response to therapy.

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Contributions

LB, MvT, and ME designed the study. LB, ME, MvdHE, and VdH were responsible for patient inclusion. FC and CV performed the experiments. FC, EE, LV, and PtH designed and analyzed the sequencing data. FC, CV and MvP designed and analyzed the hematopoietic cell culture experiments. WK performed the chromosome analysis. FC, LB and MvT drafted the manuscript. All authors critically reviewed the data and revised the final manuscript.

Appendix A. Supplementary data

An overview of studies describing MSC characteristics in MDS is available online. In addition, sequences used for RT-PCR are listed. Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.scr.2015.01.006>.

References

- Hasle, H., Baumann, I., Bergstrasser, E., et al., 2004. The International Prognostic Scoring System (IPSS) for childhood myelodysplastic syndrome (MDS) and juvenile myelomonocytic leukemia (JMML). *Leukemia* 18 (12), 2008–2014.
- Hasle, H., Niemeyer, C.M., Chessells, J.M., et al., 2003. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. *Leukemia* 17 (2), 277–282.
- Strahm, B., Nollke, P., Zecca, M., et al., 2011. Hematopoietic stem cell transplantation for advanced myelodysplastic syndrome in children: results of the EWOG-MDS 98 study. *Leukemia* 25 (3), 455–462.
- Sasaki, H., Manabe, A., Kojima, S., et al., 2001. Myelodysplastic syndrome in childhood: a retrospective study of 189 patients in Japan. *Leukemia* 15 (11), 1713–1720.
- Woods, W.G., Barnard, D.R., Alonzo, T.A., et al., 2002. Prospective study of 90 children requiring treatment for juvenile myelomonocytic leukemia or myelodysplastic syndrome: a report from the Children's Cancer Group. *J. Clin. Oncol.* 20 (2), 434–440.
- Hasle, H., Niemeyer, C.M., 2011. Advances in the prognostication and management of advanced MDS in children. *Br. J. Haematol.* 154 (2), 185–195.
- Zhang, B., Ho, Y.W., Huang, Q., et al., 2012. Altered microenvironmental regulation of leukemic and normal stem cells in chronic myelogenous leukemia. *Cancer Cell* 21 (4), 577–592.
- Morikawa, S., Mabuchi, Y., Kubota, Y., et al., 2009. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J. Exp. Med.* 206 (11), 2483–2496.
- Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., et al., 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466 (7308), 829–834.
- Sugiyama, T., Kohara, H., Noda, M., et al., 2006. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25 (6), 977–988.
- Raaijmakers, M.H., Mukherjee, S., Guo, S., et al., 2010. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* 464 (7290), 852–857.
- Schepers, K., Pietras, E.M., Reynaud, D., et al., 2013. Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. *Cell Stem Cell* 13 (3), 285–299.
- Blau, O., Baldus, C.D., Hofmann, W.K., et al., 2011. Mesenchymal stromal cells of myelodysplastic syndrome and acute myeloid leukemia patients have distinct genetic abnormalities compared with leukemic blasts. *Blood* 118 (20), 5583–5592.
- Lopez-Villar, O., Garcia, J.L., Sanchez-Guijo, F.M., et al., 2009. Both expanded and uncultured mesenchymal stem cells from MDS patients are genomically abnormal, showing a specific genetic profile for the 5q-syndrome. *Leukemia* 23 (4), 664–672.
- Flores-Figueroa, E., Montesinos, J.J., Flores-Guzman, P., et al., 2008. Functional analysis of myelodysplastic syndromes-derived mesenchymal stem cells. *Leuk. Res.* 32 (9), 1407–1416.
- Marcondes, A.M., Mhyre, A.J., Stirewalt, D.L., et al., 2008. Dysregulation of IL-32 in myelodysplastic syndrome and chronic myelomonocytic leukemia modulates apoptosis and impairs NK function. *Proc. Natl. Acad. Sci. U. S. A.* 105 (8), 2865–2870.
- Santamaria, C., Muntion, S., Roson, B., et al., 2012. Impaired expression of DICER, DROSHA, SBDS and some microRNAs in mesenchymal stromal cells from myelodysplastic syndrome patients. *Haematologica* 97 (8), 1218–1224.
- Wang, Z., Tang, X., Xu, W., et al., 2013. The different immunoregulatory functions on dendritic cells between mesenchymal stem cells derived from bone marrow of patients with low-risk or high-risk myelodysplastic syndromes. *PLoS One* 8 (3), e57470.
- Zhao, Z.G., Xu, W., Yu, H.P., et al., 2012a. Functional characteristics of mesenchymal stem cells derived from bone marrow of patients with myelodysplastic syndromes. *Cancer Lett.* 317 (2), 136–143.
- Ferrer, R.A., Wobus, M., List, C., et al., 2013. Mesenchymal stromal cells from patients with myelodysplastic syndromes display distinct functional alterations that are modulated by lenalidomide. *Haematologica* 98 (11), 1677–1685.
- Klaus, M., Stavroulaki, E., Kastrinaki, M.C., et al., 2010. Reserves, functional, immunoregulatory, and cytogenetic properties of bone marrow mesenchymal stem cells in patients with myelodysplastic syndromes. *Stem Cells Dev.* 19 (7), 1043–1054.
- Alvi, S., Shaher, A., Shetty, V., et al., 2001. Successful establishment of long-term bone marrow cultures in 103 patients with myelodysplastic syndromes. *Leuk. Res.* 25 (11), 941–954.
- Aizawa, S., Nakano, M., Iwase, O., et al., 1999. Bone marrow stroma from refractory anemia of myelodysplastic syndrome is defective in its ability to support normal CD34-positive cell proliferation and differentiation in vitro. *Leuk. Res.* 23 (3), 239–246.
- Narendran, A., Hawkins, L.M., Ganjavi, H., et al., 2004. Characterization of bone marrow stromal abnormalities in a patient with constitutional trisomy 8 mosaicism and myelodysplastic syndrome. *Pediatr. Hematol. Oncol.* 21 (3), 209–221.

- Borojevic, R., Roela, R.A., Rodarte, R.S., et al., 2004. Bone marrow stroma in childhood myelodysplastic syndrome: composition, ability to sustain hematopoiesis in vitro, and altered gene expression. *Leuk. Res.* 28 (8), 831–844.
- Roela, R.A., Carraro, D.M., Brentani, H.P., et al., 2007. Gene stage-specific expression in the microenvironment of pediatric myelodysplastic syndromes. *Leuk. Res.* 31 (5), 579–589.
- Calkoen, F.G., Brinkman, D.M., Vervat, C., et al., 2013. Mesenchymal stromal cells isolated from children with systemic juvenile idiopathic arthritis suppress innate and adaptive immune responses. *Cytotherapy* 15 (3), 280–291.
- Bronkhorst, I.H., Maat, W., Jordanova, E.S., et al., 2011. Effect of heterogeneous distribution of monosomy 3 on prognosis in uveal melanoma. *Arch. Pathol. Lab. Med.* 135 (8), 1042–1047.
- Lankester, A.C., Bierings, M.B., van Wering, E.R., et al., 2010. Preemptive alloimmune intervention in high-risk pediatric acute lymphoblastic leukemia patients guided by minimal residual disease level before stem cell transplantation. *Leukemia* 24 (8), 1462–1469.
- Mastrokolias, A., den Dunnen, J.T., van Ommen, G.B., et al., 2012. Increased sensitivity of next generation sequencing-based expression profiling after globin reduction in human blood RNA. *BMC Genomics* 13, 28.
- Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26 (1), 139–140.
- R: A Language and Environment for Statistical Computing (Vienna, Austria).
- Smyth, G., Gentleman, R., VCSDR1WH, 2005. Limma: linear models for microarray data. *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. Springer, New York, NY, USA, pp. 397–420.
- Hochberg, Y., Benjamini, Y., 1990. More powerful procedures for multiple significance testing. *Stat. Med.* 9 (7), 811–818.
- Le Blanc, K., Mougiakakos, D., 2012. Multipotent mesenchymal stromal cells and the innate immune system. *Nat. Rev. Immunol.* 12 (5), 383–396.
- Calloni, R., Cordero, E.A., Henriques, J.A., et al., 2013. Reviewing and updating the major molecular markers for stem cells. *Stem Cells Dev.* 22 (9), 1455–1476.
- Chillemi, A., Zaccarello, G., Quarona, V., et al., 2013. Anti-CD38 antibody therapy: windows of opportunity yielded by the functional characteristics of the target molecule. *Mol. Med.* 19, 99–108.
- Zhao, Z., Wang, Z., Li, Q., et al., 2012b. The different immunoregulatory functions of mesenchymal stem cells in patients with low-risk or high-risk myelodysplastic syndromes. *PLoS One* 7 (9), e45675.
- Oliveira, F.M., Lucena-Araujo, A.R., Favarin, M.C., et al., 2013. Differential expression of AURKA and AURKB genes in bone marrow stromal mesenchymal cells of myelodysplastic syndrome: correlation with G-banding analysis and FISH. *Exp. Hematol.* 41 (2), 198–208.
- Gohring, G., Michalova, K., Beverloo, H.B., et al., 2010. Complex karyotype newly defined: the strongest prognostic factor in advanced childhood myelodysplastic syndrome. *Blood* 116 (19), 3766–3769.
- Flores-Figueroa, E., Gutierrez-Espindola, G., Montesinos, J.J., et al., 2002. In vitro characterization of hematopoietic microenvironment cells from patients with myelodysplastic syndrome. *Leuk. Res.* 26 (7), 677–686.
- Minami, M., Inoue, M., Wei, S., et al., 1996. STAT3 activation is a critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line. *Proc. Natl. Acad. Sci. U. S. A.* 93 (9), 3963–3966.
- Zhang, H., Nguyen-Jackson, H., Panopoulos, A.D., et al., 2010. STAT3 controls myeloid progenitor growth during emergency granulopoiesis. *Blood* 116 (14), 2462–2471.
- Csaszar, E., Wang, W., Usenko, T., 2014. Blood stem cell fate regulation by delta-1 mediated rewiring of IL-6 paracrine signaling. *Blood* 123 (5), 650–658.
- Gunn, W.G., Conley, A., Deininger, L., et al., 2006. A crosstalk between myeloma cells and marrow stromal cells stimulates production of DKK1 and interleukin-6: a potential role in the development of lytic bone disease and tumor progression in multiple myeloma. *Stem Cells* 24 (4), 986–991.
- Ara, T., Declerck, Y.A., 2010. Interleukin-6 in bone metastasis and cancer progression. *Eur. J. Cancer* 46 (7), 1223–1231.
- Dayar, J.M., Choy, E., 2010. Therapeutic targets in rheumatoid arthritis: the interleukin-6 receptor. *Rheumatology (Oxford)* 49 (1), 15–24.
- Melief, S.M., Geutskens, S.B., Fibbe, W.E., et al., 2013. Multipotent stromal cells skew monocytes towards an anti-inflammatory interleukin-10-producing phenotype by production of interleukin-6. *Haematologica* 98 (6), 888–895.
- Song, L., Webb, N.E., Song, Y., et al., 2006. Identification and functional analysis of candidate genes regulating mesenchymal stem cell self-renewal and multipotency. *Stem Cells* 24 (7), 1707–1718.
- Looyenga, B.D., Resau, J., MacKeigan, J.P., 2013. Cytokine receptor-like factor 1 (CRLF1) protects against 6-hydroxydopamine toxicity independent of the gp130/JAK signaling pathway. *PLoS One* 8 (6), e66548.
- Kass, D.J., Yu, G., Loh, K.S., et al., 2012. Cytokine-like factor 1 gene expression is enriched in idiopathic pulmonary fibrosis and drives the accumulation of CD4+ T cells in murine lungs: evidence for an antifibrotic role in bleomycin injury. *Am. J. Pathol.* 180 (5), 1963–1978.
- Raval, A., Tanner, S.M., Byrd, J.C., et al., 2007. Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. *Cell* 129 (5), 879–890.
- Qian, J., Yao, D.M., Lin, J., et al., 2010. Methylation of DAPK1 promoter: frequent but not an adverse prognostic factor in myelodysplastic syndrome. *Int. J. Lab. Hematol.* 32 (1 Pt 2), 74–81.
- Wu, X., Liu, W., Tian, Y., et al., 2011. Aberrant methylation of death-associated protein kinase 1 CpG islands in myelodysplastic syndromes. *Acta Haematol.* 125 (4), 179–185.
- Claus, R., Hackanson, B., Poetsch, A.R., et al., 2012. Quantitative analyses of DAPK1 methylation in AML and MDS. *Int. J. Cancer* 131 (2), E138–E142.
- Karlic, H., Herrmann, H., Varga, F., et al., 2014. The role of epigenetics in the regulation of apoptosis in myelodysplastic syndromes and acute myeloid leukemia. *Crit. Rev. Oncol. Hematol.* 90 (1), 1–16.
- Glaubach, T., Robinson, L.J., Corey, S.J., 2014. Pediatric myelodysplastic syndromes: they do exist! *J. Pediatr. Hematol. Oncol.* 36 (1), 1–7.
- Hirabayashi, S., Flotho, C., Moetter, J., et al., 2012. Spliceosomal gene aberrations are rare, coexist with oncogenic mutations, and are unlikely to exert a driver effect in childhood MDS and JMML. *Blood* 119 (11), e96–e99.
- Thanopoulou, E., Cashman, J., Kakagianne, T., et al., 2004. Engraftment of NOD/SCID-beta2 microglobulin null mice with multilineage neoplastic cells from patients with myelodysplastic syndrome. *Blood* 103 (11), 4285–4293.
- Kerbaui, D.M., Lesnikov, V., Torok-Storb, B., et al., 2004. Engraftment of distinct clonal MDS-derived hematopoietic precursors in NOD/SCID-beta2-microglobulin-deficient mice after intramedullary transplantation of hematopoietic and stromal cells. *Blood* 104 (7), 2202–2203.
- Groen, R.W., Noort, W.A., Raymakers, R.A., et al., 2012. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* 120 (3), e9–e16.
- Walkley, C.R., Olsen, G.H., Dworkin, S., et al., 2007. A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. *Cell* 129 (6), 1097–1110.