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Chapter 3.

Despite differential gene expression profiles pediatric MDS derived mesenchymal stromal cells display normal 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 micro-environment. Here, we extensively studied the mesenchymal stromal cells (MSC) derived from children with MDS.

MSC were expanded from 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 MSCs expressed increased levels of *DKK3*, a protein associated with decreased apoptosis. RAEB(t)-MDS revealed increased *CRLF1* and decreased *DAPK1* expression. This pattern has been associated with transformation in hematopoietic malignancies. Genes reported to be differentially expressed in adult MDS-MSCs did not differ between MSCs of pediatric MDS and controls.

An altered mRNA expression profile, associated with cell survival and malignant transformation, of MSCs derived from children with MDS strengthen 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.

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, 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, 2003) Survival has increased from 30 to 60% since hematopoietic stem cell transplantation (HSCT) is applied.(Strahm, 2011; Sasaki, 2001; Woods, 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, 2011; Hasle, 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, 2012b) In children, no conclusive data is yet available.

Mesenchymal stromal cells (MSC) have been identified as supporting cells of hematopoietic stem cells (HSC) *in vivo* and *in vitro* (Morikawa, 2009; Mendez-Ferrer, 2010; Sugiyama, 2006b) and linked to disease, as aberrant MSC function was shown to contribute to the pathophysiology of malignant disorders in murine models.(Raaijmakers, 2010; Schepers, 2013) Characteristics of MSCs from adult MDS patients have been extensively studied focusing on cytogenetic and molecular abnormalities(Blau, 2011; Lopez-Villar, 2009; Flores-Figueroa, 2008) as well as gene and protein expression.(Marcondes, 2008; Flores-Figueroa, 2008; Santamaria, 2012) In addition, abnormal immunomodulation(Wang, 2013b; Zhao, 2012b; Marcondes, 2008) as well as decreased hematopoietic support(Zhao, 2012b; Ferrer, 2013) by MSCs has been reported in MDS. However, these data remain conflicting with other studies reporting no abnormalities in stromal function.(Flores-Figueroa, 2008; Klaus, 2010; Alvi, 2001) Differences in results may be explained by a variety in MSC expansion protocols and experimental set-up, but also by the heterogeneity of the disease.(Aizawa, 1999) Studies reporting on (cyto)genetics and function of MDS-MSCs have been summarized in the 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 MSCs derived from an MDS patient with trisomy 8,(Narendran, 2004) a study using stroma cells of 7 MDS patients (Borojevic, 2004), and a gene-expression analysis of the stromal compartment by the same research group.(Roela, 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 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. (Hasle, 2003) 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, 2013a) Briefly, bone-marrow mononuclear cells (MNC) 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 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 passage 2-3 and 5-7, respectively. All but anti-CD105 (Ancell Corporation Bayport, MN) antibodies were derived from Becton Dickinson Biosciences (BD), San Diego, CA, USA. Culture supernatant was collected after reaching 80% confluency at passage 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 chromosome 7 and 8 was performed on MSCs from patients with known monosomy 7 or trisomy 8 using the

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; MSC: mesenchymal stromal cells; CB: cord blood; Bu: busulphan; Cy: cyclofosfamide; Mel: melphalan; rATG: rabbit anti-thymocyte globulin; Flu: fludarabine; Thio: thiotepa; Treo: treosulphan; ARA-C: cytosine arabinoside; n.a.: not applicable.

following probes: Vysis LSI D7S486/CEP7 and LSI IGH/LSI MYC, CEP8, (Abbott Laboratories, Abbott Park, IL,USA).(Bronkhorst, 2011)

Chimerism analysis

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

Immunomodulatory assays

The effect of MSCs (30 Gy irradiated) on proliferation of peripheral blood (PB) MNC obtained from adult bloodbank donors (100 000 cells/well) after stimulation with phytohemagglutinin (PHA 2 μg/mL) was analyzed at MSC : PBMC ratios of 1:5 and 1:40. MSC and PB-MNC were co-cultured in RPMI P/S, 10% (v/v) fetal calf serum (FCS) for 5 days with the addition of ^{3}H-thymidine (1 μCi/well; Perkin Elmer, Wellesley, MA, USA) for the last 16 hours 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 (DC). 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 μg/mL Beckman-Coulter, Marseille, France) to generate mature DC. 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 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 (HPC) were performed to determine the supportive capacity of MSCs for HPC maintenance and differentiation. Therefore, HPC were isolated from 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 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 $10x10^{3}$ $CD34^{+}$ cells at a CD34 : MSC ratio of 1:5 for flow cytometry analysis. Half of

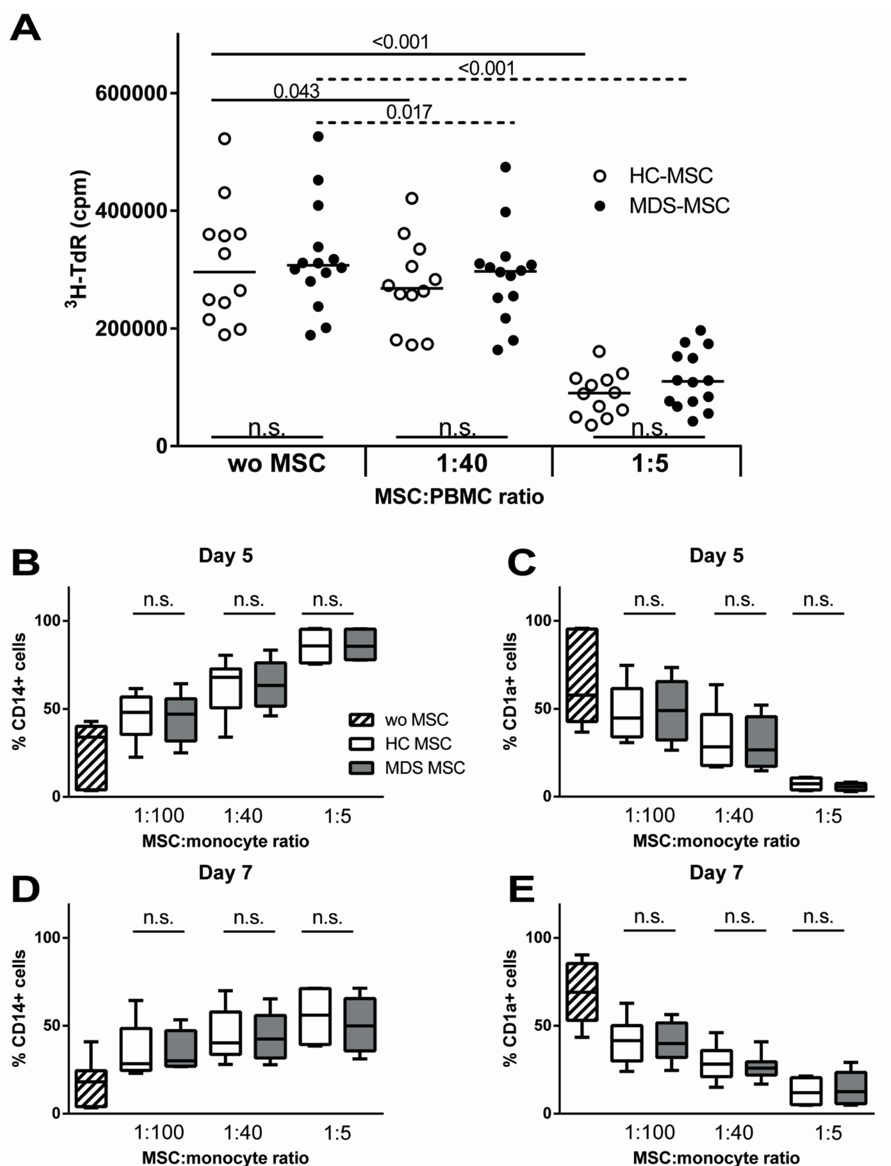

Figure 1. *Immunomodulation by MDS-MSCs.* A. Both healthy control (HC, n=6) and MDS-MSCs (n=5 of which RAEB/RAEBt n=3 and RCC n=2) significantly suppressed PHA-induced PBMC (n=4 healthy adults) proliferation at MSC : PBMC ratios 1:40 and 1:5. No significant differences were observed between MDS patients (black circles) and controls (white circles). wo MSCs: without MSCs. B-E. MSCs suppressed the differentiation of monocytes ($CD14^+$ cells) towards dendritic cells ($CD1a^+$ cells) in a dose-dependent manner (hatched boxes: no MSCs added). No significant differences were observed between HC (white boxes) and MDS-MSCs (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.

the culture medium was refreshed with the addition of growth factors on day 4, 7 and 11. Proliferation (day 7) and differentiation (day 7 and 14) was assessed using ^{3}H-thymidine during the last 16 hours or flow cytometry, respectively. Antibodies used for flow cytometry were anti-CD34-PE, anti-CD45-FITC, anti-CD38-Percpc5.5, anti-CD45-Percpc5.5, 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 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 HPC in CFU-assays, MSCs (30 000 or 150 000 per dish) were added to freshly purified HPC (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 passage 2-3 using a Qiagen RNeasy Minikit (Qiagen, Hilden, Germany). mRNA was profiled using Deep-SAGE sequencing using Illumina technology.(Mastrokolias, 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).

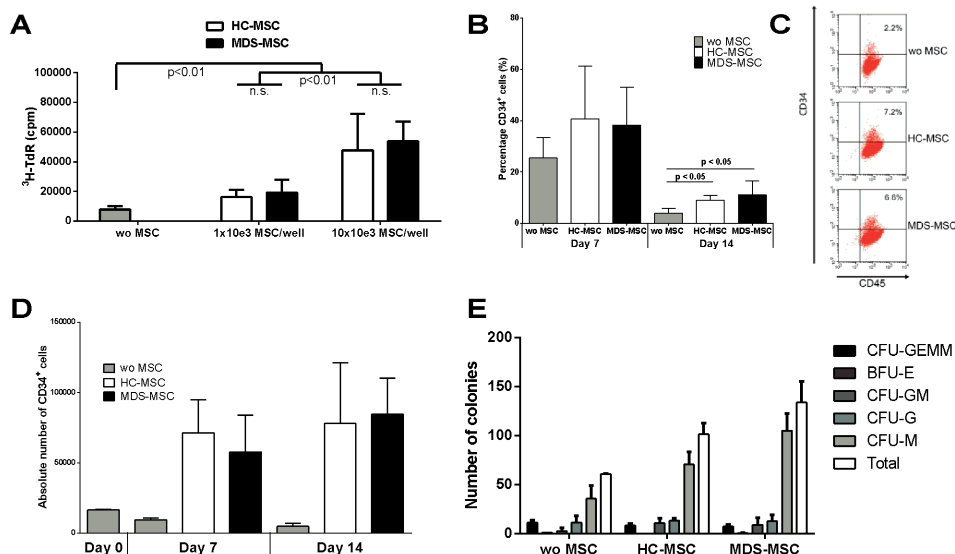

Figure 2. *MDS-MSCs support maintenance and differentiation of hematopoietic progenitor cells (HPC).* A. $CD34^+$ cells (500 cells) of HSCT donors were cultured in the absence (wo MSCs) or presence of MSC (CD34 : MSC ratios 1:2 and 1:20) obtained from MDS patients or healthy controls (HC) in the presence of SCF and Flt3-L. Proliferation of HPC at day 7 was assessed by ^{3}H-thymidine incorporation. B-C. $CD34^+$ expression declined overtime, but MSCs supported the expansion of HPC ($CD34^+$ cells) and a higher percentage of $CD34^+$ cells was 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 $10x10^3$ HPC. 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 HPC decreased significantly in the absence of MSCs, the absolute number of HPC increased in co-cultures with MSCs ($CD34^+$: MSC ratio 1:5). Data depicted in A, B and D represent at least 2 independent experiments of 5 HC-MSCs and 8 MDS-MSCs (grey 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 MSCs co-culture. wo MSCs: without MSCs. Bars depict the mean with standard deviation. *P*-values were calculated using Mann-Whitney tests. n.s.: not significant.

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, 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, 2010; R Development Core Team, 2012; Smyth, 2005) Correction for multiple testing was performed according to Benjamini and Hochberg.(Hochberg, 1990) Adjusted p-values <0.05 were considered statistically significant.

Results

Expansion and characterization of MSCs

MSCs were successfully cultured from bone-marrow of all patients and controls. Expanded MSCs expressed CD73, CD90 and CD105, whereas the cells did not express lineage markers (Supplementary Figure S1A). MSCs differentiated towards adipocytes and osteoblasts when cultured in culture media supporting these distinct directions of differentiation (Figure 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 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, 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-MSC and healthy control (HC-)MSC (Figure 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 DC. This process further progressed from day 5 to 7 during maturation of DC 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 day 5 and 7 at various MSC : monocyte ratios (Figure 1B-E). No differences between both groups were observed.

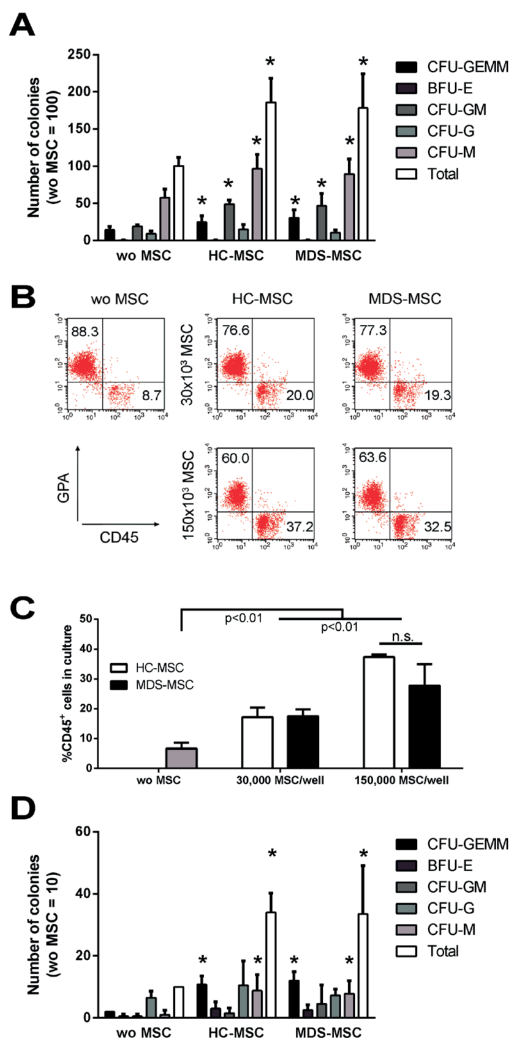

Figure 3. *MSCs of MDS patients support colony formation in CFU-assays.* A-C. MSCs (30 000 cells, or 150 000 cells when indicated) of healthy controls (HC-MSCs), of MDS patients (MDS-MSCs) or no MSC (wo MSCs) were added to freshly isolated HPC ($CD34^+$ cells, 500 cells) of HSCT donors at initiation of colony forming unit assays (CFU-assay). Cultures contain exogenous SCF, GM-CSF, IL-3 and EPO. Addition of MSCs support the increase 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 is 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 HPC 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 MSCs: 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.

RAEB(t)-MSC and RCC-MSC 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 HPC.(Mendez-Ferrer, 2010) The effect of MSCs of pediatric MDS patients and healthy children on expansion and differentiation of $CD34^+$ HPC 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 (Figure 2A). The percentage of cells expressing CD34 declined over time, but the level of this decline is significantly less in cultures containing MSCs (Figure 2B-C). In co-cultures of HPC with MSCs, CD38 expression, associated with activation but also with loss of stemcellness,(Calloni, 2013; Chillemi, 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 HPC 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 (Figure 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 HSC that have been cultured in the absence of MSCs (Figure 2E). This is in accordance with the increased $CD34^+$ numbers that were observed (Figure 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, $50x10^3$ $CD34^+$ cells were seeded on confluent MSC layers and cultured for 3-5 weeks without the addition of growth factors. When cultured in

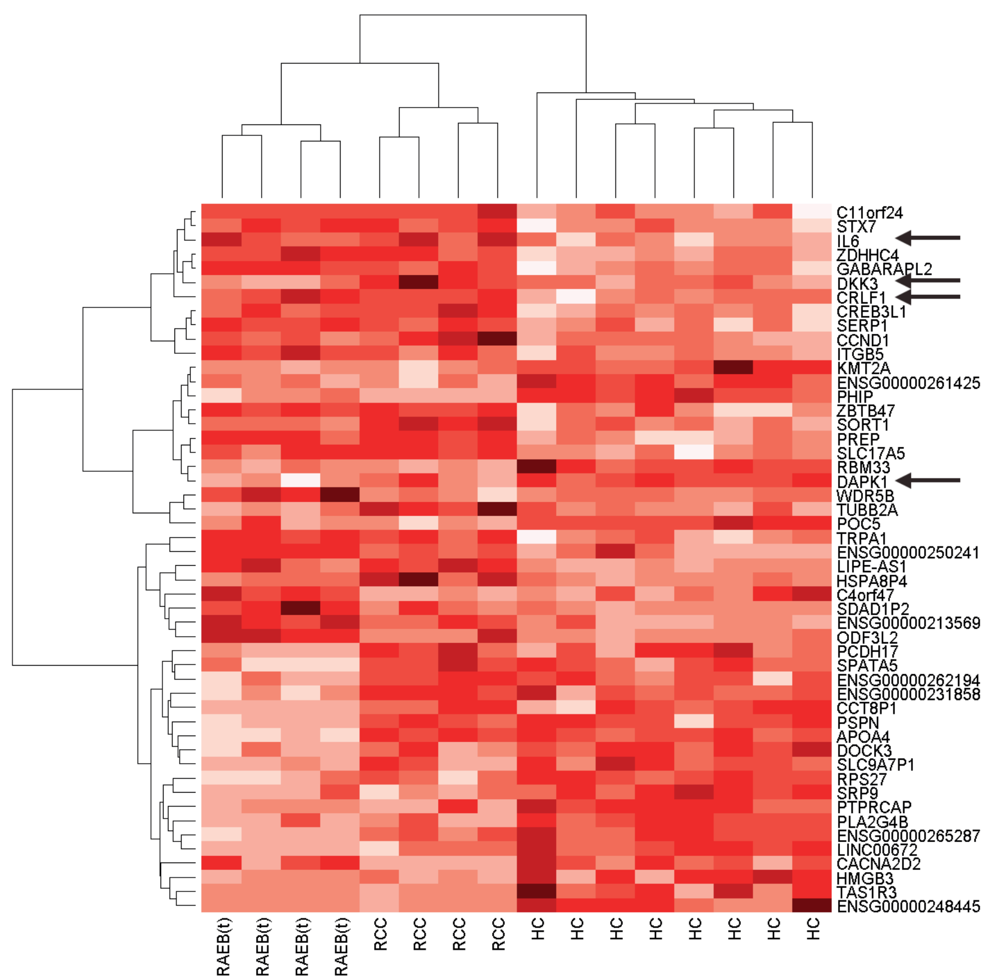

Figure 4. *Heat map depicting clustering of MDS derived MSCs.* Gene expression was analyzed using LIMMA software. MSCs of different groups (RAEB(t)-MDS, RCC-MDS and healthy controls (HC)) showed hierarchical clustering in a heat map of differentially expressed genes. Color intensity of the squares correlates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

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) (Figure 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) (Figure S2B).

Support of colony formation

To study the direct influence of MSCs on colony formation by freshly isolated HPC, MSCs ($30x10^3$ or $150x10^3$ 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 (Figure 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 was increased in the non-adherent cell population (colonies) harvested at day 14 from cultures containing MSCs (Figure 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 HPC 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 was significantly increased in comparison with cultures without MSCs (Figure 3D). In conclusion, HC-MSCs and MDS-MSCs have similar effects on colony formation *in vitro*.

Gene and protein expression

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 formally not exclude the possible existence of biologically relevant differences between MDS-MSCs en 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 $15.9x10^6$ reads (range $9.5x10^6$ – $30.6x10^6$) was obtained. Between 59.3% and 68.4% (median 65.6%) of reads were 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 MSCs (Figure 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 higher expressed in RCC-MSCs compared to HC-MSC (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* expression were increased (p=0.009 and p=0.048, respectively). Differential expression of *IL6*, *DKK3*, *DAPK1* and *CRLF1* was confirmed by RT-PCR (Figure 5A). In addition, the IL-6 concentration in culture supernatants of MSCs from 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; Figure 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 (Figure 5C). IL-6 concentration in MDS-MSCs culture supernatant was, although lower than in MSC samples generated

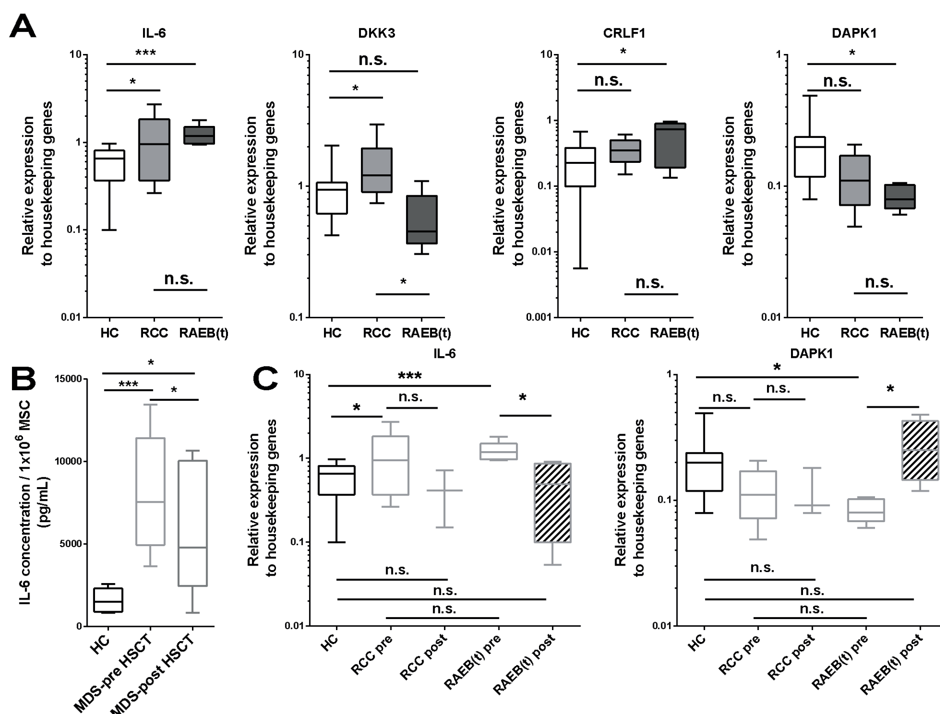

Figure 5. *Differential mRNA expression by MSCs of MDS patients.* A: Aberrant gene expression detected by Deep-SAGE was confirmed by RT-PCR. *IL-6* expression was increased in MSCs of both RCC and RAEB(t) MDS patients at diagnosis compared to MSCs of healthy controls (HC). In contrast, *DKK3* was specifically 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-MSCs n=10, RCC-MSCs n=7 and RAEB(t)-MSCs 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 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.

from bone-marrow taken pre-HSCT, still significantly higher than in supernatant of HC-MSC (Figure 5B). *DKK3* and *CRLF1* expression in MSCs expanded after HSCT from RCC and RAEB(t) patients, respectively, was not significantly altered compared to MDS-MSCs before HSCT or to HC-MSCs (data not shown).

Potential candidate genes based on their reported differentially expression in adult MDS-MSCs, i.e. *AURKA*, *AURKB*, *SCF*, *G-CSF* and *GM-CSF* (Zhao, 2012a; Santamaria, 2012; Oliveira, 2013; Ferrer, 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, 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, 2012b; Schepers, 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* expression 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, 2012b; Narendran, 2004) In contrast, other studies in adults did not show differential *IL6* expression between MDS patients and healthy controls.(Zhao, 2012b; Klaus, 2010; Flores-Figueroa, 2002; Flores-Figueroa, 2008) *IL6* has been described to increase myeloid differentiation via *STAT3* activation and support multiple myeloma cell growth and survival.(Minami, 1996; Zhang, 2010; Csaszar, 2013; Gunn, 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 disease.(Ara, 2010; Dayer, 2010) Suppression of monocyte to dendritic cells differentiation is dependent on IL-6. (Melief, 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, 2006; Looyenga, 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, 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, 2007; Qian, 2010; Wu, 2011; Claus, 2012; Karlic, 2013) We demonstrate a similar expression profile in our MSCs, with expression in RCC-MDS being similar to HC-MSC, 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 HPC 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.*(Santamaria, 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.(Glaubach, 2014; Hasle, 2004; Hirabayashi, 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.(Le, 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 of 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 HPC with MDS patient or healthy control derived MSCs.

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

In conclusion, our data show that the gene expression profile is different in MSCs 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 MSCs after allogeneic HSCT is an argument favoring the latter possibility. Induced abnormalities in the MSCs by dysplastic cells might be targets to sustain response to therapy.

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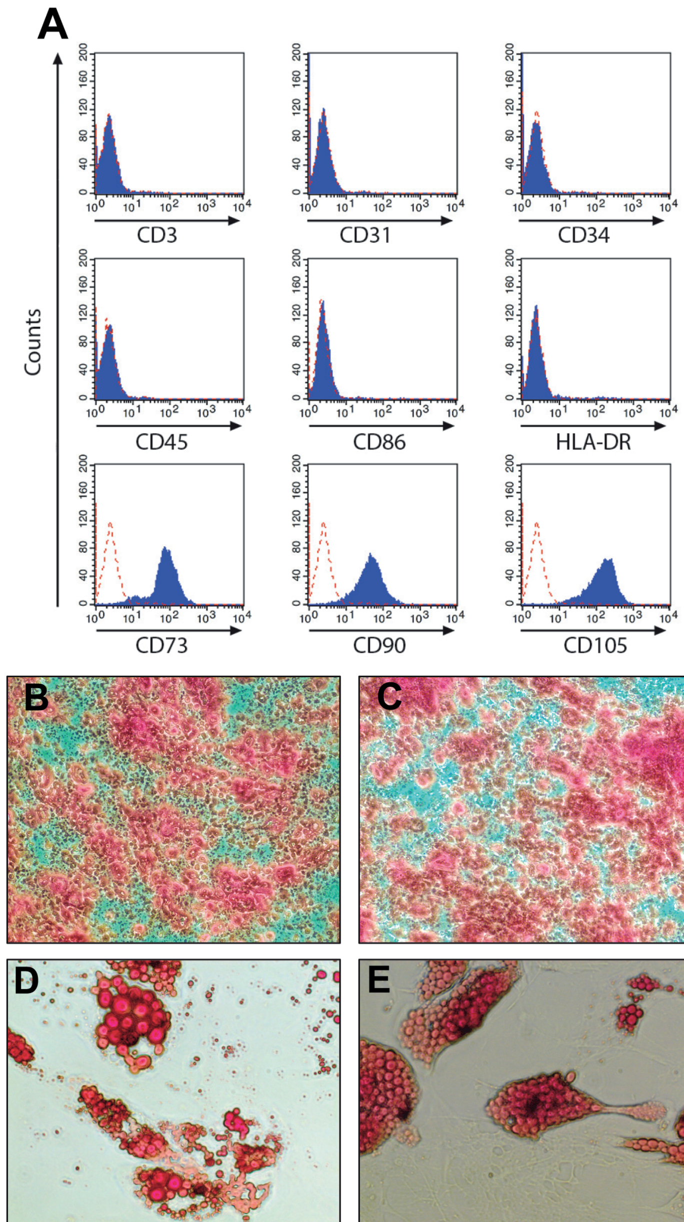
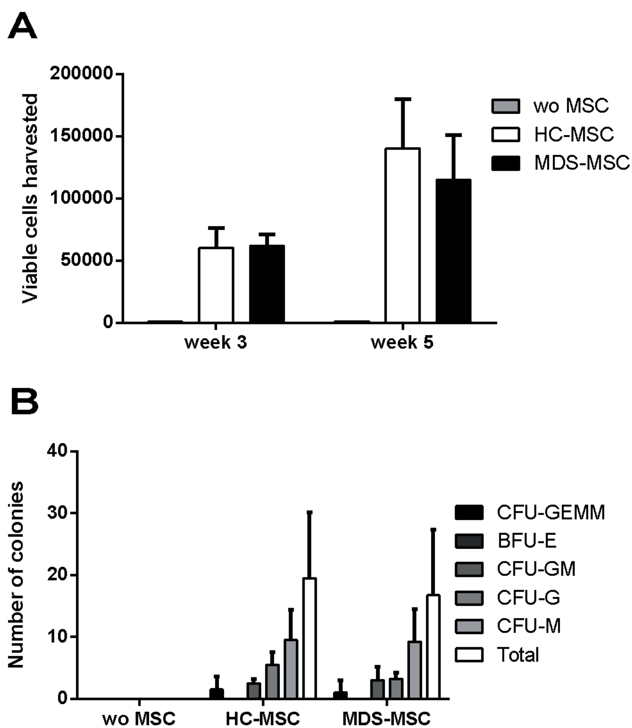
Supplementary data



Supplementary Figure 1. *Phenotype and differentiation capacity of MDS-MSC.* A: MSC of MDS patients (*e.g.* UPN MDS015, passage 2) express the characteristic phenotype of MSC: CD73$^+$, CD90$^+$ and CD105$^+$ and negative for the indicated lineage-specific markers. Red line indicates the staining intensity obtained with a negative isotype-matched control. B-E: MSC of healthy controls (B, D HC012, passage 5) and MDS patients (C, E MDS002, passage 7) differentiate towards osteoblasts (B, C Alizarine red staining, magnification 10x) and adipocytes (D, E Oil-Red-O staining, magnification 40x).



Supplementary Figure 2. *MSC of MDS patients maintain hematopoietic cells in the absence of growth factors.* CD34^{+} cells (HPC, 50 000 cells) of HSCT donors were seeded on a confluent layer of MSC (white bars: healthy control (HC)-MSC, black bars: MDS-MSC) or in wells without (wo) MSC (grey bars) and cultured for 3 to 5 weeks. A. Viability of non-adherent cells obtained after harvesting was assessed by trypan blue staining. No viable cells were present in culture conditions without MSC. B. Non-adherent cells harvested after 3 weeks of co-culture with healthy control (HC)-MSC (n=2) or MDS-MSC (n=4) were tested in CFU-assays (1500 cells/dish) to investigate their differentiation capacity. Results are depicted as the mean and standard deviation.

Supplementary Table 1. Studies describing characteristics and function of MDS derived MSC in adults

Author	Ref	Year	Study set-up	MSC characterization	Cytogenetic abnormalities	Protein expression	Gene expression	Immuno-modulation	Hematopoietic support	Technique for analysis of hematopoiesis
Blau	(164)	2011	43 MDS 51 AML + 36 controls	Yes	5 out of 43 MDS using karyotype	NA	NA	NA	NA	NA
Lopez-Villar	(165)	2009	36 MDS + 15 controls	Yes, decreased growth	all cases using array-CGH	low CD105	NA	NA	NA	NA
Flores Figueroa	(166)	2008	16 MDS + 6 controls	negative selection; then characterized	8 out of 12 using karyotype	IL-1b increased; IL-6 a.o. similar	NA	NA	no differences	5 week culture
Blau	(212)	2007	18 MDS 13 AML 7 controls	Yes	7 out of 16 using karyotype	NA	NA	NA	NA	NA
Flores Figueroa	(213)	2005	11 MDS + 5 controls	Negative selection; then characterized	5 out of 9 using karyotype; all MDS MSC hypodiploid	NA	NA	NA	NA	NA
Song	(214)	2012	22 MDS + 7 controls	Yes	15 out of 22 using karyotype	NA	NA	NA	NA	NA
de Oliveira	(189)	2013	60 MDS	yes, decreased growth	18/60 using karyotype	NA	AURKA AURKB increased (espe-cially in abnormal karyotype)	NA	NA	NA
Marcondes	(167)	2008	13 MDS + 6 controls	Yes	NA	Increased TNF and IL32	IL-32 increased	NA	NA	NA
Lubkova	(215)	2011	5 MDS + 7 controls	Yes	NA	decreased VCAM1	NA	NA	NA	NA
Santamaria	(168)	2012	33 MDS + 25 controls	Yes	NA	NA	Dicer Drosha SBDS decreased; effect on miRNA	NA	NA	NA
Flores Figueroa	(191)	2002	25 MDS + 8 controls	Yes	NA	TNF-a increased IL-6 normal	NA	NA	NA	NA

Author	Ref	Year	Study set-up	MSC characterization	Cytogenetic abnormalities	Protein expression	Gene expression	Immuno-modulation	Hematopoietic support	Technique for analysis of hematopoiesis
Hirayama	(216)	1993	8 AA + 7 MDS + 9 controls	passage 0	NA	large variation	large variation	NA	NA	
Aanei	(217)	2012	20 MDS + 8 controls	yes, decreased growth	NA	NA	decrease in CD29, CD49e, CD44, CD31, CD106	NA	NA	NA
Aanei	(218)	2011	9 MDS + 4 controls	use passage 1 CD73 enrichment; in MDS more morphological changes + decrease proliferation	NA	increase in focal adhesion proteins	NA	NA	NA	NA
Wang	(169)	2013	31 MDS + 8 controls	yes	NA	NA	NA	low risk MDS less suppres-sion of DC maturation, TGF-b most important	NA	NA
Zhao	(170)	2012	29 MDS + 10 controls	yes	normal karyotype	more IL-6; less TGFb1 (for low risk MDS) HGF	NA	strong save from apopto-sis, less PHA suppression, less induction of T-reg (low risk MDS only)	NA	NA
Zhao	(188)	2012	14 MDS + 8 controls	yes + clonal expansion	normal karyotype	NA	decreased SCF, G-CSF, GM-CSF; increased IL-6	MLR + PHA; impaired sup-pression;	decreased support	5 week culture
Han	(219)	2007	15 RA-MDS + 12 controls	yes, but negative selection	normal karyotype	NA	TGFb1+3 + FasL decrease; TGFb2 increase	impaired suppression of PHA + MLR; less suppres-sion of cell cycle; increase saving from apoptosis	NA	NA

Author	Ref	Year	Study set-up	MSC characterization	Cytogenetic abnormalities	Protein expression	Gene expression	Immuno-modulation	Hematopoietic support	Technique for analysis of hematopoiesis
Ferrer	(171)	2013	20 MDS + 6 controls	yes, altered growth morphology and differentiation	NA	decreased adhesion proteins and SDF1a	SDF1 ANG1 SCF lower	normal sup-perssion of PHA induced proliferation	reduced support by MDS MSC	CAFC assays 6 weeks
Varga	(220)	2007	10 MDS + 15 controls	Yes	NA	NA	NA	NA	decreased support	CAFC assays 6 weeks
Tennant	(221)	2000	16 RA + 7 RARS + 5 RAEB + 2 RAEBt + 2 CMML	passage 0	NA	NA	Increased Epo, G-CSFr TNFr TPOr decrease IL-1b	NA	decreased in MDS	5-7 week culture
Flores Figueroa	(222)	2012	various	direct biopsy IHC	NA	increased CXCL12	NA	NA	NA	NA
Coutinho	(223)	1990	10 MDS 4 controls	passage 0	NA	NA	NA	NA	similar support	7 week culture
Soenen-Cornu	(224)	2005	12 MDS + n controls	Yes	FISH no abnormalities	NA	NA	NA	supportive but no controls	2 and 5 week culture
Klaus	(172)	2010	13 MDS + 20 controls	acquired during culture in 4 patients and 1 control	yes	TNFa, IL1b, IL-6 VEGF SDF1a similar	NA	similar PHA suppression	NA	NA
Alvi	(173)	2001	103 MDS + 12 controls	use of BM biopsy not MNC	NA	NA	NA	NA	normal support	2 week culture
Aizawa	(174)	1999	11 RA + 12 controls	passage 0	NA	NA	NA	NA	decreased support, but large variation between MDS patients	5 week culture

MDS: myelodysplastic syndrome; MSC: mesenchymal stromal cells; AML: acute myeloid leukemia; NA not applicable; CGH: comparative genomic hybridisation; AA: aplastic anemia; MLR: mixed lymphocyte reaction; RA: refractory anemia; CAFC: cobblestone area-forming cells; RARS: RA with ring sideroblasts; RAEB: RA with excess of blasts; RAEBt: RAEB in transformation; CMML: chronic myelomonocytic leukemia; IHC: immunohistochemistry.

Supplementary Table 2. Studies describing characteristics and function of MDS derived MSC in children

Author	Ref	Year	Study set-up	MSC characterization	Cytogenetic abnormalities	Protein expression	Gene expression	Immuno-modulation	Hematopoietic support	Technique for analysis of hematopoiesis
Narendran	(175)	2004	case-report	yes	constitutional trisomy 8	Increased TGF-b, VEGF, IL-6, LIF; decreased SCF	NA	NA	increased stromal support of HPC and leukemic cells	NA
Borojevic	(176)	2004	7 MDS patients	yes	NA	NA	OSF2, SPARC, COL1A2, THBS1 increased	NA	decreased maturation and absolute numbers	NA
Roela	(177)	2007	6 MDS 3 controls	yes	NA	NA	different patterns of endocytosis and protein secretion related genes	NA	NA	NA

MDS: myelodysplastic syndrome; MSC: mesenchymal stromal cells; HPC hematopoietic progenitor cells; NA not applicable.

Supplementary Table 3. Primer design for RT-PCR

CRLF1-forward	CCAGAGAAACCCGTCAACAT
CRLF1-reverse	TCCTGGCCATACCACCTAAG
DAPK1-forward	AGCCCGTCATGATCTACCAG
DAPK1-reverse	CTCCGAGTGAGGAGGTTCAG
IL-6-forward	GAAAGCAGCAAAGAGGCACT
IL-6-reverse	TTTCACCAGGCAAGTCTCCT
DKK3-forward	TGGGGAAATGTGGAGAAGAG
DKK3-reverse	GAGCAACACTGCTGGATGAA
Dicer1-forward	GAGGCGTGTCTTGAAAAAGC
Dicer1-reverse	AGTTAGGACTGCGGAAAGCA
DROSHA-forward	AATGGATGCGCTTGAAAAAT
DROSHA-reverse	TGCATGCCCTCCTTTATTTC
CXCL12-forward	AGAGCCAACGTCAAGCATCT
CXCL12-reverse	CTTTAGCTTCGGGTCAATGC
HPRT1-forward	TGACACTGGCAAAACAATGCA
HPRT1-reverse	GGTCCTTTTCACCAGCAAGCT
GAPDH-forward	GGCCTCCAAGGAGTAAGACC
GAPDH-reverse	AGGGGAGATTCAGTGTGGTG