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## Autophagy in normal hematopoiesis and leukemia

Folkerts, Hendrik

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# 04.

## **Erythroid progenitors from patients with low-risk myelodysplastic syndromes are dependent on the surrounding micro environment for their survival**

Hendrik Folkerts, Carin L.E. Hazenberg, Ewout J. Houwerzijl,  
Fiona A.J. van den Heuvel, André B. Mulder,  
Johannes J.L. van der Want and Edo Vellenga

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## Abstract

To investigate whether the type of programmed cell death of myelodysplastic erythroid cells depends on their cellular context, we performed studies on cells from patients with low-risk myelodysplastic syndromes. We compared erythroid cells (and their precursor cells) from the mononuclear cell fraction with those from the hematons fraction, which are compacted complexes of hematopoietic cells surrounded by their own micro-environment. In directly fixed materials, erythroblasts exhibited signs of autophagy with limited apoptosis (<3%) based on ultrastructural characteristics and immunogold labeling for activated caspase-3. After 24 h in culture, myelodysplastic erythroblasts exhibited a significant increase in apoptosis ( $22 \pm 7\%$  vs.  $3 \pm 2\%$ ,  $p=0.001$ ). In contrast, the myelodysplastic erythroblasts from the hematons fraction did not exhibit an increased tendency toward apoptosis after culture ( $7 \pm 3.3\%$  vs.  $1.8 \pm 2.3\%$ ), which was in line with results for normal bone marrow cells. The same dependency on the micro-environment was noted for immature erythroid progenitor cells. Myelodysplastic hematomas exhibited distinct numbers of erythroid burst-forming units in association with an extensive network of stromal cells, whereas small numbers of erythroid burst-forming units were generated from the myelodysplastic mononuclear cells compared with normal mononuclear cells ( $10.2 \pm 9$  vs.  $162 \pm 125$ ,  $p=0.001$ ). Co-culture of erythroid myelodysplastic cells in the presence of growth factors (vascular endothelial growth factor, leukemia inhibitory factor) or on the MS-5 stromal layer did not restore the expansion of erythroid precursor cells. These data indicate that surviving myelodysplastic erythroid progenitors become more vulnerable to programmed cell death when they are detached from their own micro-environment.

## Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal stem cell disorders characterized by ineffective and dysplastic hematopoiesis [1,2]. Especially in low-risk MDS, such as refractory anemia and refractory anemia with ringed sideroblasts, increased programmed cell death (PCD) of bone marrow hematopoietic cells has been described [1–6]. This might be an important mechanism that accounts for the typical clinical findings of a hypercellular bone marrow and peripheral blood cytopenias. In particular, enhanced apoptosis has been reported in MDS [1,2]. However, recent studies indicate that alternative types of cell death also take place. Non-apoptotic PCD was reported in megakaryocytes of patients with MDS and was characterized by the absence of chromatin condensation and caspase-3 and caspase-8 activation [3]. In immature erythroid cells, enhanced autophagy was observed without evidence of apoptosis [7]. There may be several reasons for the differences in the type of PCD in various MDS studies, including differences in patient characteristics, cell-specific behavior, detection techniques, and bone marrow sample handling and preparation. Moreover, cells are capable of switching between various types of PCD depending on their cellular context. For instance, inhibition of the apoptotic machinery can trigger a switch from apoptosis to necrosis or autophagy [8]. Loss of mitochondrial transmembrane potential plays an important role in these events, and the intensity of the stimulus and the cellular context often determine which type of cell death occurs [9]. On the basis of these findings, it is conceivable that the recognized differences in the type of PCD in MDS might be related to whether cells are studied in *in vitro* culture assays or in the context of their own micro-environment. Previous studies have reported that hematons can be isolated from the bone marrow light-density fraction [10,11]. These hematons are compact hematopoietic complexes containing several cell lineages, including mesenchymal cells, endothelial cells, and hematopoietic progenitor cells. In these hematons, a large number of erythroblasts are located within their own micro-environment. We therefore decided to determine whether the type of PCD of MDS erythroid precursors is dependent on their cellular context. To answer this question, we compared MDS erythroid precursors from the mononuclear cell (MNC) fraction with those from the hematons fraction. Erythroid cells directly prepared from bone marrow hematons and MNC fractions exhibited limited numbers of apoptotic cells. Instead, the ultrastructural results were compatible with the presence of autophagy. However, when MNCs from MDS bone marrow

were cultured for 24 hours *in vitro*, a relatively large proportion of apoptotic erythroid cells appeared, an increase that was not observed in erythroid cells from the hematopoietic fraction.

## Materials and Methods

### Patients

Bone marrow of low-risk MDS patients (n=17) and healthy controls (n=10) was aspirated from the iliac crest (**Table 1; suppl. Table 1**) The median age of the control group was 55 years (range 20-70) and consisted of allogeneic stem cell donors. The diagnosis of MDS was made according to the World Health Organization (WHO) classification. The international prognostic scoring system (IPSS) was used to define prognostic risk groups [12]. Patients were placed into three groups with good, intermediate, and poor-risk cytogenetics. Transfusion dependency was defined if patients needed one or more units of red blood cells per month. The institutional review board of the University Hospital Groningen approved the study protocol. All patients gave informed consent.

### Electron microscopy

#### *Bone marrow sample preparation.*

Fresh bone marrow cells were washed in RPMI-1640 (BioWhittaker Europe, Verviers, Belgium), pelleted, and subsequently fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 24 hrs at 4°C. Cells were dehydrated, osmicated, and embedded in Epon 812 according to routine procedures. Semi-thin sections (0.5 µm) stained with toluidine blue were inspected using light microscopy to select erythroblasts. The ultrastructure of erythroid precursors was studied in both hematopoietic and the MNC fraction from fresh bone marrow aspirate. Hematopoietic were prepared as previously described [6,10]. Bone marrow hematopoietic and mononuclear cell fractions were also studied after 24 hrs of culture in RPMI-1640 with 5% fetal calf serum (FCS). A total of 100 erythroblasts per sample were examined.

#### *Immunogold labeling.*

Bone marrow mononuclear cells and hematopoietic were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde, pH 7.4 at 4°C for 30 minutes. For cryosectioning, the bone marrow samples were embedded in 5% gelatine and

frozen in liquid nitrogen. Thin sections (80 nm) were cut at 100°C for immune-electron microscopy. Cryosections were picked up in a 2.3 M saturated sucrose solution. The sections were then labeled with rabbit anti-caspase-3 antibody (Cell Signaling Technology, Danvers, MA). Sections were mounted in 1.5% methylcellulose containing 0.4% uranyl acetate and examined in detail with electron microscopy (Philips 201, Eindhoven, the Netherlands).

*Ultrastructural definitions of programmed cell death and autophagy.*

Apoptosis and autophagy/autophagic cell death were defined according to recent recommendations of the Nomenclature Committee on Cell Death [13]. Briefly summarized, apoptosis and autophagy are distinct morphological processes. Autophagy requires the presence of cytoplasmic double membrane vacuoles containing degenerating cytoplasmic organelles or cytosol. Autophagolysosomes are identified by electron microscopy as vacuoles that contain both molecular markers of lysosomes (lysosome-associated membrane protein-2, LAMP-2) and molecular markers of organelles (mitochondria) or morphological remnants of organelles (iron deposits are considered as mitochondrial remnants) [7].

***In vitro* culture assay**

The burst forming unit-erythroid (BFU-E) assay was performed in duplicate in methylcellulose (MethoCult H4230, StemCell Technologies, Grenoble, France) supplemented with 1 U EPO (Cilag Eprex, Brussels, Belgium) as previously described [14]. After 14 days BFU-E were counted. In a selected number of experiments Epo was combined with Leukemia inducing factor (LIF), 20ng/ml, Sigma, Zwijndrecht, the Netherlands and Vascular endothelial growth factor (VEGF, 20ng/ml), R&D Systems, Abingdon, U.K. This assay was also used for studying the erythroid potential of a hematon to propagate erythroid precursors, which was added as single unit to the methylcellulose. For the long-term cultures, MNCs were plated in 12-well plates pre-coated with MS5 stromal layer as previously described [15]. Cells were expanded in LTC medium (alpha-minimal essential medium supplemented with heat-inactivated 12.5% fetal calf serum (Sigma, Zwijndrecht, the Netherlands), heat-inactivated 12.5% horse serum (Sigma), penicillin, 2 mM glutamine, 1 uM hydrocortisone (Sigma) supplemented with Epo and stem cell factor (SCF, 10 ng/ml). Cultures were kept at 37°C and 5% CO<sub>2</sub>. The cell number was counted weekly in conjunction with the differential. For the suspension culture assay a similar set-up was used, but without the MS5 stromal layer. Finally a limited number of experiments were performed by

pre-coating the flask with fibronectin (5 µg/ml, Sigma-Aldrich, Zwijndrecht, the Netherlands) followed by the suspension culture assay.

### Flow cytometry analysis

The bone marrow-derived MNCs of MDS patients (n=5) and healthy subjects (n=4) were obtained by density gradient centrifugation. Nucleated cells were stained for 30 min at 20°C with the following antibodies: PCy5 (Lineage) labeled: CD2, CD3, CD4, CD7, CD8, CD19, CD20, CD56, CD123 (eBioscience, Hertfordshire, United Kingdom) and CD10 (Biolegend, Uithoorn, the Netherlands). FITC labeled CD235A (DakoCytomation, Glostrup, Denmark) and PCY7 labeled CD34 (Becton Dickinson, Breda, the Netherlands) were used. Lineage depleted cells were sorted for CD235A<sup>+</sup>, CD34<sup>+</sup> or CD34<sup>-</sup>CD235A<sup>-</sup> cell fractions on a MoFlo XDP or MoFlo Astrios cell sorter (DakoCytomation, Carpinteria, CA, USA). Data were analyzed using FlowJo 7.6.1.

### Real-time PCR

Quantitative RT-PCR was performed to examine the mRNA expression levels of ATG5, ATG7, Beclin1, LC3, GATA1, GATA2 and GLYA. Total RNA was isolated from 1x10<sup>5</sup> cells using RNeasy kit (Qiagen, Venlo, the Netherlands). RNA was reverse transcribed with iScript reverse Transcription kit (Bio-Rad, Veenendaal, the Netherlands). Obtained cDNA was real-time amplified in iQ SYBR Green Supermix (Bio-Rad) with the CFX connect Thermocycler (Bio-Rad). RPL27 was used as a housekeeping gene. The primer sequences are:

ATG5	FOR: 5'-GGGCCATCAATCGGAAAC-3' REV: 5'-AGCCACAGGACGAAACAG-3'
Beclin1	FOR: 5'-CATGCAATGGTGGCTTTC-3' REV: 5'-TCTCCACATCCATCCTGTAG-3'
ATG7	FOR: 5'-CGTTGCCACAGCATCATCTTC-3' REV: 5'-TCCCATGCCTCCTTCTGGTTC-3'
LC3	FOR: 5'-CGCACCTTCGAACAAAGAGTAG-3' REV: 5'-AGCTGCTTCTCACCTTGTATC-3'
GATA1	FOR: 5'-ACACTGTGGCGAGAAATGC-3' REV: 5'-AGATGCCTTGGCGTTTCGAG-3'
GATA2	FOR: 5'-AGCAAGGCTCGTTCCTGTTC-3' REV: 5'-GTCGGTTCTGCCATTCATC-3'
GLYA	FOR: 5'-ACCCTCCAGAAGAGGAAACC-3' REV: 5'-CAGTCGGCGAATACCGTAAG-3'
RPL27	FOR: 5'-TCCGGACGCAAAGCTGTCATCG-3' REV: 5'-TCTTGCCCATGGCAGCTGTAC-3'

## Statistical analysis

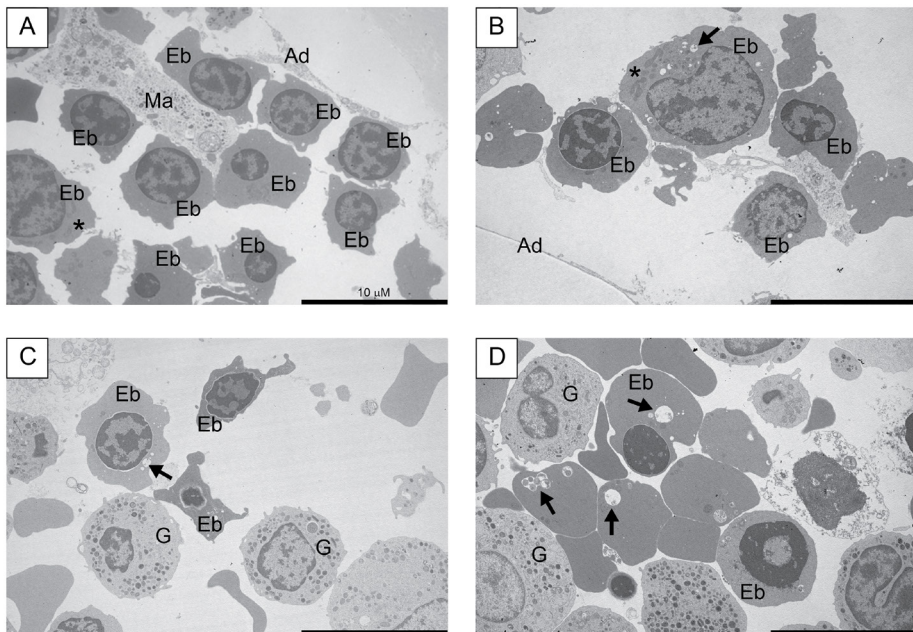
Unpaired two-sided student's test or Mann-Whitney test were used to calculate statistical differences. A P-value of <0.05 was considered significant.

## Results

To evaluate whether cell processing methods and interaction with the microenvironment affects the type of PCD, erythroblasts from MNC fraction and bone marrow hematons were ultra-structurally analyzed after being freshly prepared and after culturing for 24 hrs. Representative results are depicted in **Figure 1**. Compared to immediately fixed erythroblasts, the 24 hrs cultured cells of healthy controls (n=4) showed a higher level of apoptosis ( $10.2 \pm 3.3\%$  of erythroblasts vs  $1.4 \pm 2.6\%$  of erythroblasts,  $p < 0.003$ ), and significantly more cytoplasmic vacuolization ( $58.0 \pm 10.3\%$  vs.  $21.0 \pm 11.4\%$ ,  $p < 0.001$ ) following the culture period (**Fig. 2**). Also, normal bone marrow hematons (n=4) displayed a slightly higher percentage of erythroblasts showing apoptosis ( $7.2 \pm 6\%$  vs.  $1.8 \pm 4.0\%$ ,  $p = 0.1$ ) after culturing, and a strongly elevated level of cells showing cytoplasmic vacuolization ( $14.5 \pm 7.6\%$  vs.  $63.2 \pm 13.0\%$ ,  $p < 0.001$ ) compared to directly prepared samples (**Fig. 2**). The MDS cells were studied in a similar manner (**Fig. 1B**). After culturing for 24 hrs, a significantly higher fraction of MDS erythroid cells (n=7) displayed features of apoptosis ( $22.4 \pm 7.8\%$  vs.  $3.4 \pm 2.4\%$ ,  $p < 0.001$ ) than from the directly fixed samples, based on typical ultra-structural characteristics and immunogold labeling for activated caspase-3, as illustrated in **Fig. 3**. This coincided with a decrease in the percentage of morphologically intact erythroblasts ( $36 \pm 19\%$  vs.  $12 \pm 12\%$  [median 6.5 (6-30%)],  $p = 0.08$ ). The percentage of erythroblasts showing cytoplasmic vacuolization remained relatively unchanged during culture ( $56.6 \pm 14.8\%$  vs.  $54.3 \pm 16.4\%$ , ns). In contrast, the hematons of the same MDS patients showed a modest increase in erythroblasts undergoing apoptosis after culture ( $7.0 \pm 3.3\%$  vs.  $1.8 \pm 2.3\%$ ,  $p < 0.01$ ), whereas the fraction of erythroblasts showing cytoplasmic vacuolization increased strongly ( $61.8 \pm 16.9\%$  vs.  $30.8 \pm 11.3\%$ ,  $p = 0.004$ ). These findings indicate that erythroblasts display distinct signs of apoptosis if the cells are disconnected from the micro-environment. To determine whether the same phenomena occur with erythroid precursor cells, we cultured MNC and hematon cells in the BFU-E assay. MNC cells from MDS patients (n=8) showed significantly reduced BFU-E colony formation after 14 days of culture compared to normal MNC ( $10.2 \pm 9$  vs.  $162 \pm 125$ ,  $p < 0.001$ ). From the same MDS patients, the hematon fractions were also cultured in the BFU-E assay and displayed distinct erythroid colony formation in association

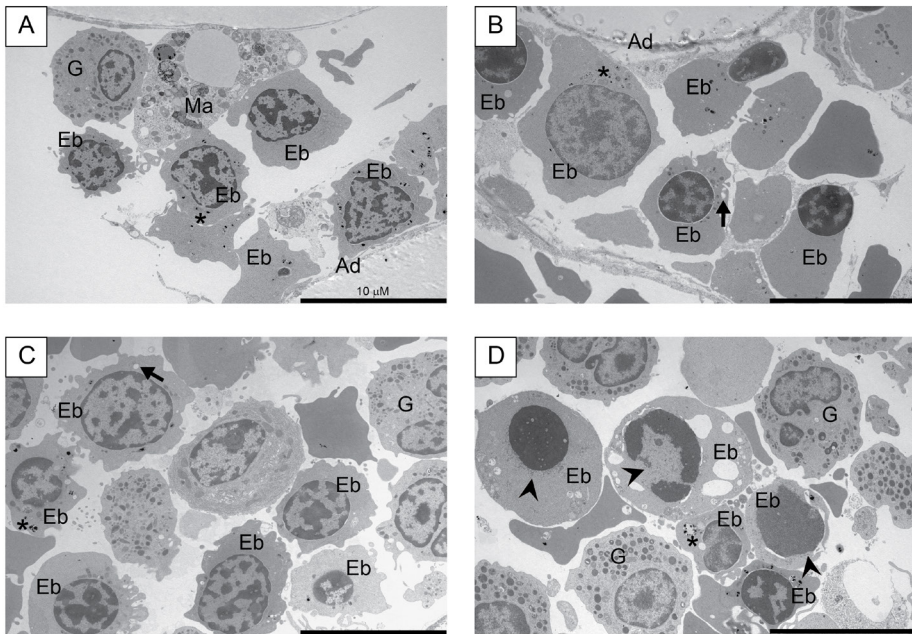


**Figure 1** In **1A** and **1B**, examples of ultrastructural features of erythroblasts are shown within their micro-environment by using hematons or in suspension using MNC fractions from normal bone marrow cells and MDS before and after short-term culture. Erythroblasts show a marked difference in structural changes between the hematons and MNC fractions of healthy donors and MDS patients.



**Figure 1A** Ultrastructure of normal hematons before and after short-term culture (**A** and **B**) and MNC fraction before and after short-term culture (**C** and **D**). **A**. Erythroblasts (Eb) are closely opposed to an adipocyte membrane (Ad), with an intruding macrophage in between (Ma). Spherical erythroblast (Eb) nuclei show homogeneously distributed chromatin with increased opacity, electron dense cytoplasm with few mitochondria (asterisk) and occasionally vacuoles (arrows). **B**. After short-term culture the hematons show similar structural characteristics, slightly more prominent vacuoles (arrows). **C**. Normal MNC erythroblasts show typical successive stages of maturation with increasing nuclear density and cytoplasmic vacuoles (arrows). Some granulocytes (G) can also be seen. **D**. Normal MNC after 24 hours in culture show examples of apoptosis of erythroblasts and some debris containing vacuolar inclusions (arrows) that are slightly larger and more numerous compared to the vacuoles in the uncultured condition (G: granulocyte, Ma: macrophage; Eb: erythroblasts). Bar is 10  $\mu$ m.

with an extensive network of stromal cells (**Fig. 4**). We were able to expand these immature and mature erythroid precursor cells of the hematons fraction for a period of 3-6 weeks. To confirm that the expanded erythroid cells of the hematons fraction belonged to the dysplastic clone, expanded erythroid cells of RARS patients were studied by iron staining. A high number of ring-sideroblasts could be demonstrated indicating that the expanded cells emerging from the hematons fraction were derived from the dysplastic clone (data not shown). In contrast to normal erythroid precursor cells, the connection of MDS erythroid cells with their micro-environment appears to be particularly critical for their



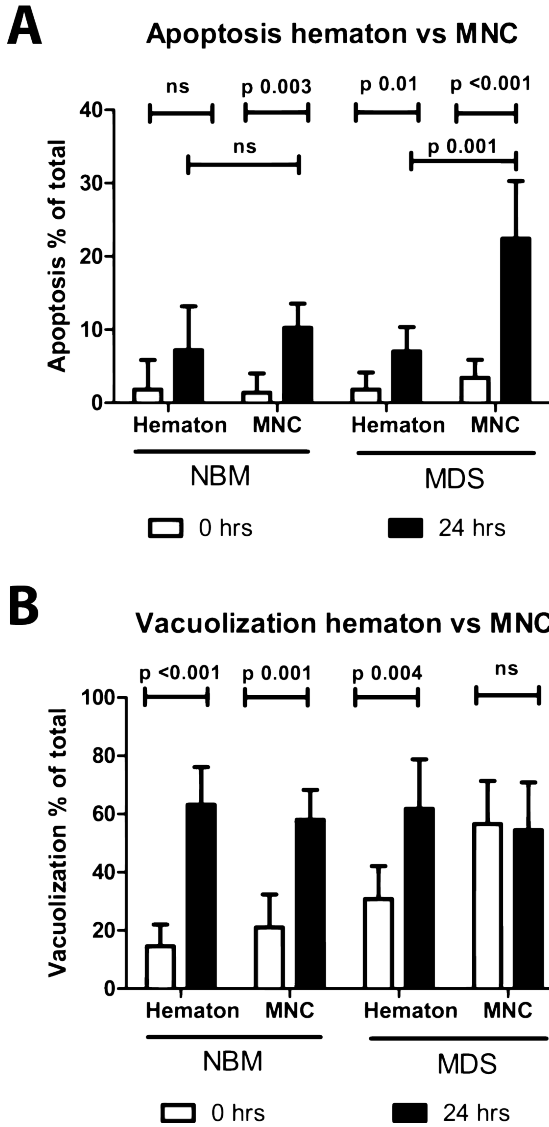
**Figure 1B** Ultrastructure of MDS hematons before and after 24 hours in culture (**A** and **B**) and MNC fraction of the same patient before and after short-term culture (**C** and **D**). **A**. Several erythroblasts (Eb) are grouped between two adipocytes (Ad), the cytoplasm of the erythrocytes shows a characteristic finding in MDS: iron deposits in mitochondria or the remnants of the mitochondria following mitophagy (asterisks). **B**. The cellular niche in the hematons, next to an adipocyte (Ad), shows various phases of maturation and increasing density of the cytoplasmic matrix. Mitochondria are still present following culture and most of them contain iron deposits. **C**. An overview shows various cell types surrounding erythroblasts in successive stages of maturation and a mature erythrocyte. Next to the erythroblasts are a plasma cell and thrombocytes. **D** Following culture, erythroblasts show apoptotic features like pyknotic nuclei with condensed chromatin and mitochondria with clusters of iron (asterisk). (G: granulocyte, Ma: macrophage, Eb: erythroblast, Ad: adipocytes). Bar is 10 µm.

survival since the addition of additional growth factors potentially produced by the micro-environment (VEGF and LIF) could not restore the BFU-E colony formation;  $5.0 \pm 4.2$  and  $3.7 \pm 3.0$  ( $n=3$ ) with respectively Epo vs. Epo plus VEGF and LIF. To determine whether this can be modelled *in vitro*, we cultured MNC cells of MDS patients in suspension with Epo and SCF or on an MS5 stromal layer in the presence of these growth factors. As depicted in **Fig. 5**, normal MNCs ( $n=5$ ) expanded most prominently if the cells were cultured on the MS5 stromal layer ( $6.4 \pm 6.0$  fold increase vs.  $33.8 \pm 22$ , at day 14,  $p=0.02$ , **Fig. 5A**). Moreover the differential count demonstrated that 90% of the expanded cells belonged to the erythroid lineage. A similar design was used for MDS cells and showed a limited expansion of the erythroid precursor cells in the suspension culture assay with Epo and SCF ( $1.2 \pm 0.7$  fold after 14 days) which could be

augmented by culturing the cells on MS5 stromal layer ( $2.9 \pm 1.7$  fold,  $p=0.04$ ). However, the degree of expansion was significantly less as compared to normal MNCs (**Fig. 5B**). Differential counts of the expanded MDS cells after 14 days of culture demonstrated predominantly erythroid cells (>80%). The expansion of erythroid cells on the MS5 stromal layer could not be ascribed to activation of the fibronectin receptors (VLA4 and VLA5). If the MS5 stromal layer was replaced by a fibronectin under layer, the expansion factor was almost comparable to the results obtained with the suspension culture assays  $1.5 \pm 0.39$ ,  $n=2$ ). Finally, we determined whether the triggering of the apoptotic process in the MDS erythroblasts might be related to an altered expression of the autophagy genes, since a reduced expression of autophagy genes can be a trigger for apoptosis [8]. We therefore sorted bone marrow of normal subjects and MDS patients into a CD235A<sup>+</sup>CD34<sup>-</sup> fraction. The accuracy of the sorting was controlled by analyzing the GATA-1 and GATA-2 expression by qPCR and demonstrated a low expression of GATA2 and high expression in GATA-1 in the more differentiated fraction (CD235A<sup>+</sup>CD34<sup>-</sup>; **Suppl. Fig. 1**). Subsequently, several essential autophagy genes (ATG5, ATG7, Beclin1, LC3) were studied in normal ( $n=4$ ) and MDS subfractions ( $n=5$ ). but no significant differences in expression were found (**Suppl. Fig.1**).

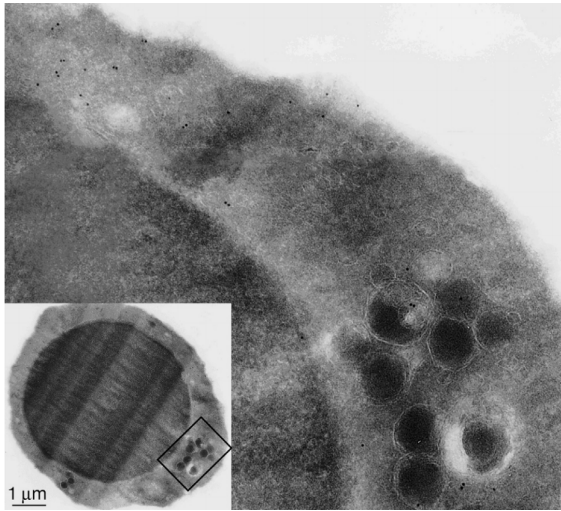
## Discussion

Although different genetic defects have recently been identified in patients with MDS, a common signature of all these abnormalities is the disturbed erythroid differentiation in combination with a tendency toward cell death [1,2,16,17]. Initially, it was proposed that apoptosis was the dominant type of cell death, but more recent studies have indicated that apoptosis is not the sole mechanism [1,3,6,7]. MDS erythroid precursor cells, in particular, exhibit signs of enhanced autophagy, whereas the other hematopoietic lineages can exhibit other types of PCD. It has not yet been resolved whether the increase in autophagy should be considered a cell survival mechanism or a type of cell death emanating from affected mitochondria. In the present study, we used an *in vitro* experimental design to determine whether erythroid cells embedded in their micro-environment are less liable to undergo PCD. Freshly isolated MDS erythroid cells from the MNC or hematopoietic fraction exhibited signs of autophagy that converted to apoptosis after a short culture period in suspension. Moreover, erythroid precursors belonging to the dysplastic clone could be expanded if the cells were cultured within their own micro-environment. Apparently, MDS erythroid cells are more dependent on pro-survival signals from the micro-environment than are normal erythroid cells.

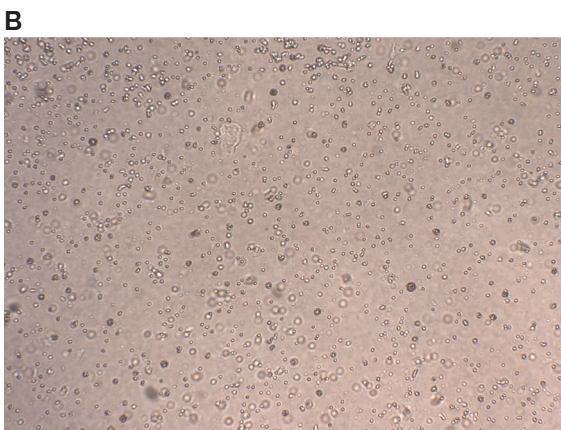
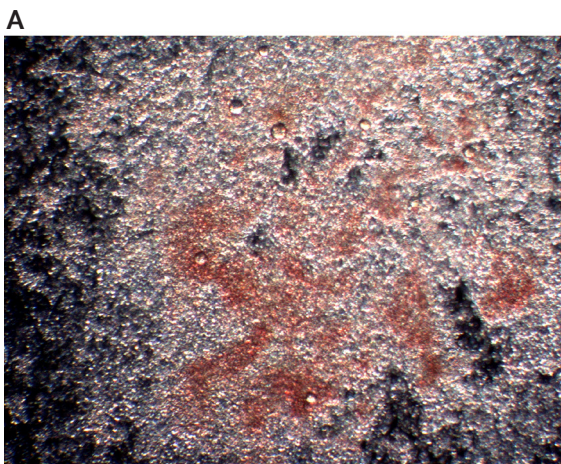


**Figure 2** Bone marrow mononuclear cells (MNC) or hematon were directly fixed or cultured for 24 hours. Ultrastructural studies of erythroblasts were performed with regard to apoptosis (A), and vacuolization (B). NBM: normal bone marrow; MDS: myelodysplastic syndrome. The % of apoptosis of normal MNC erythroblasts demonstrated an increase during culture ( $1.4 \pm 2.4$  vs.  $10.2 \pm 3.3$ ) but less compared to MDS-MNC ( $3.4 \pm 2.4$  vs.  $22.4 \pm 7.8$ ).





**Figure 3** Immuno-electron microscopy of MDS erythroblasts 24 hrs in culture. Immunogold labeling shows presence of active caspase-3 in an erythroblast from the MNC fraction

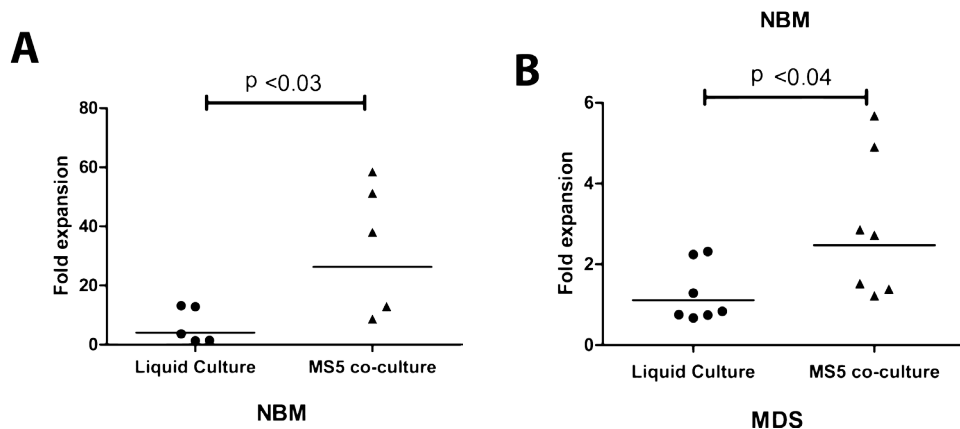


**Figure 4** Bone marrow mononuclear cells (MNC) and hematons of the same patient were cultured in the BFU-E assay during 14 days. The MNC fraction displays no BFU-E colony formation after 14-days of culture (A) while the hematons display expanding erythroid precursors (B). Photos were taken with 10x magnification from 24 wells plates.

This is probably linked to the intrinsic properties of the cells, as gene profiling of MDS CD34<sup>+</sup> cells has revealed alterations in favor of apoptotic programming without alterations in the major autophagy genes [5,18]. Several studies have reported an increased number of apoptotic cells in patients with MDS. These studies included material from bone marrow biopsies or *in vitro* culture assays. In both situations, the handling procedure or the *in vitro* detachment from the micro-environment might be an important determinant of the type of cell death observed. An interesting finding is that the MDS erythroid progenitors could be propagated *in vitro* over a longer period if the cells were cultured from the hematone fraction. Hematones are compact particles from the bone marrow containing hematopoietic, endothelial, and mesenchymal cells and might therefore be considered hematopoietic niches within a stromal framework [10,11]. So far, it has been difficult to develop humanized mouse models for MDS. The injected cells have limited capacity to expand *in vivo*, which is likely related to the altered cell death programming [19]. Recently, progress was reported after co-injection of MDS cells with mesenchymal cells [20]. In view of our findings, it is likely that pro-survival signals are also provided in this system by mesenchymal and other cells from the micro-environment, resulting in better survival of the hematopoietic stem and progenitor cells.

### **Acknowledgements**

We are grateful to dr B. Giepmans for reviewing the manuscript.



**Figure 5** Normal bone marrow (A) or MDS (B) mononuclear cells (MNC) were cultured in suspension or on the MS5 stromal layer. After 14 days of culture, the fold expansion was determined in conjunction with differential count.

No.	Age	Sex (M/F)	WHO	Hb (mM)	WBC (x 10 <sup>9</sup> /L)	Platelets (x 10 <sup>9</sup> /L)	Transfusion dependency	Karyotype analysis	IPSS
1	68	F	RA	7.1	3.1	75	Yes	Del 20q	0.5
2	54	F	RA	5.4	1.0	108	Yes	Normal	0.5
3	63	M	RA	7.9	5.2	342	No	+8	0.5
4	80	F	RARS	5.6	9.5	490	Yes	Normal	0
5	67	M	RARS	7.6	4.2	115	Yes	-Y	0.5
6	65	M	RARS	5.0	3.2	199	Yes	+8	1.0
7	84	F	RARS	5.9	2.4	68	No	-5	1.0
8	72	M	RARS	4.6	3.1	261	Yes	Normal	0.5
9	68	F	RARS	6.5	2.8	70	No	-Y	0.5
10	64	M	RA	5.3	2.4	10	Yes	-Y	1.0
11	72	F	RAEB1	7.1	2.1	94	No	-Y	1.0
12	75	M	RARS	5.5	8.0	470	No	Normal	0
13	73	F	RA	5.0	5.5	101	No	Normal	0
14	65	M	RA	6.0	6.7	100	No	Normal	0
15	87	F	RARS	5.2	3.0	109	Yes	+19	0.5
16	64	F	RARS	7.0	2.5	160	No	Normal	0.5
17	80	M	RARS	7.2	6.6	57	No	t(5;6)(p14;p22)	1.0

**Table 1** Characteristics of studied MDS patients. Abbreviations: F, female; Hb, hemoglobin, IPSS, international prognostic scoring system; M, male; RA, refractory anemia; RARS, Refractory anemia with ringed sideroblasts; RAEB: refractory anemia with excess of blasts.

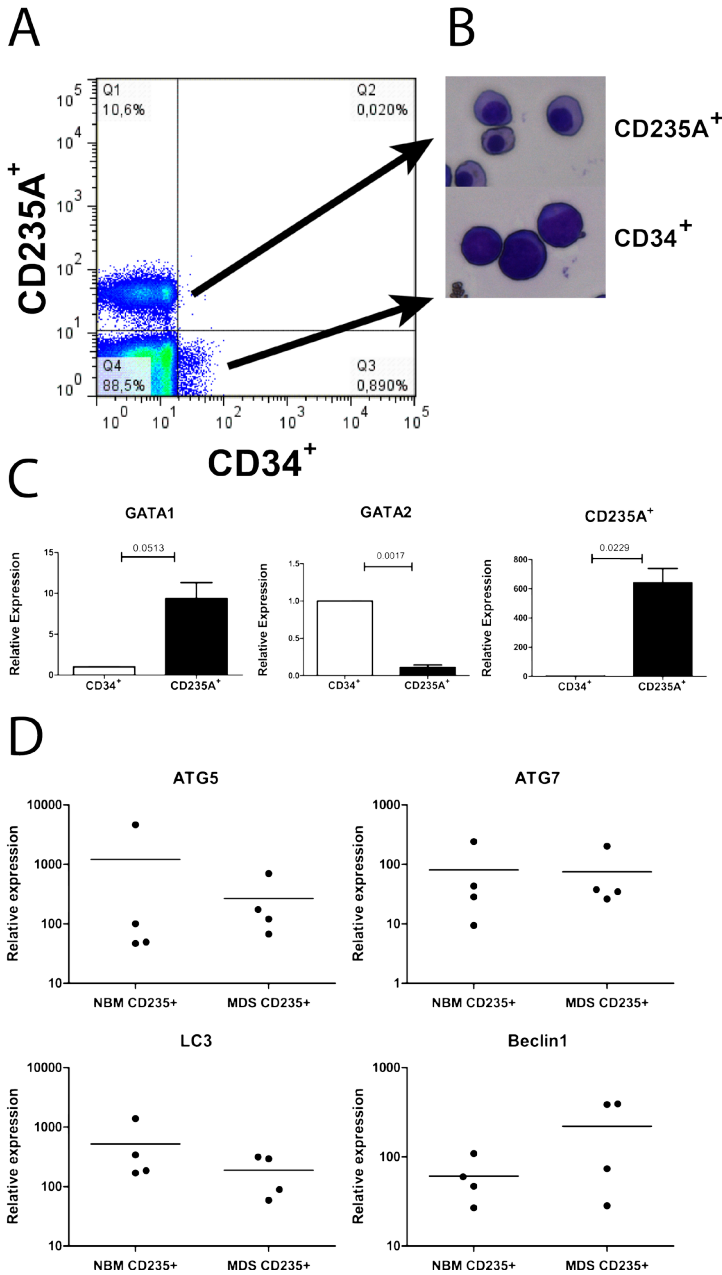
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Patients no.	EM	BFU-E (Hematon/MNC)	MS5	Fibron	BFU-E with LIF/VEGF
1	+				
2	+				
3	+				
4	+				
5	+				
6	+	+			
7	+	+			
8		+	+		
9		+	+		
10		+	+		
11		+	+		
12		+	+		
13		+	+	+	
14			+	+	
15					+
16					+

**Supplemental Table 1** Studies performed with bone marrow cells of different myelodysplasia patients. Patient number corresponds with patient number in table 1. EM: electron microscopy; BFU-E colony from hematon or mononuclear cell fraction; MS5: experiments performed with MS5 stromal layer; BFU-E colony formation with Epo in the absence and presence of VEGF and LIF. Studies performed with (fibron)ectin as under layer for suspension culture assay.



**Supplementary Figure 1 A:** Representative flow cytometry plot. The total mononuclear cell fractions (MNC) from normal bone marrow (NBM) was lineage depleted and subsequently stained for CD235A and CD34. CD235A<sup>+</sup>CD34<sup>-</sup> cells were sorted by flow cytometry. **B:** Cytopspins from sorted cell populations stained with May Grünwald Giemsa (MGG). **C:** qPCR for GATA1, GATA2 and CD235A on sorted cell populations. cDNA levels were normalized against RPL27, and values are shown relative to the expression in the CD235A<sup>+</sup>/CD34<sup>-</sup> fraction. **D:** qPCR for ATG5, Beclin1, ATG7 and LC3 in sorted CD235<sup>+</sup>CD34<sup>-</sup> cell fraction of MDS (N=4) or normal bone marrow (N=5).

