Ectopic expression of C/EBPα and ID1 is sufficient to restore defective neutrophil development in low-risk myelodysplasia

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background

In patients with myelodysplasia, a general defect in the multipotent stem-cell compartment results in disturbed proliferation and differentiation of the erythroid, megakaryocytic and myeloid lineages. Although a number of genetic defects in myelodysplastic progenitor cells have been described, the intracellular signaling pathways underlying aberrant regulation of myelopoiesis remain relatively undefined.

Design and Methods

Here, an *ex vivo* differentiation system was used to selectively screen for molecules improving defective hematopoiesis in myelodysplastic CD34$^+$ progenitor cells.

Results

Bone marrow-derived CD34$^+$ cells isolated from patients with low-risk myelodysplastic syndrome showed impaired capacity to proliferate and differentiate as well as increased levels of apoptosis. In an attempt to improve the expansion and differentiation of the myelodysplastic CD34$^+$ progenitors, cells were treated with the p38MAPK pharmacological inhibitor SB203580, or retrovirally transduced to ectopically express active protein kinase B (PKB/c-akt), or the transcriptional regulators STAT5, C/EBPα or ID1. Whereas treatment of progenitors with SB203580, PKB or STAT5 did not enhance neutrophil development, ID1- and C/EBPα-transduced cells exhibited increased granulocyte/macrophage colony formation. Furthermore, ectopic expression of C/EBPα resulted in improved neutrophil maturation.

Conclusions

These data suggest that targeting the ID1 and C/EBPα transcriptional regulators may be of benefit in the design of novel therapies for low-risk myelodysplasia.

Key words: myelodysplastic syndrome, myeloid, ID1, C/EBPα, hematopoiesis.

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Introduction

Myelodysplastic syndromes (MDS) are defined as clonal stem-cell disorders characterized by ineffective hematopoiesis and with an increased risk of transformation to acute myeloid leukemia (AML).[1] Leukemic transformation from normal stem cells is believed to be a multistep process during which a normal hematopoietic stem cell acquires multiple genetic and epigenetic abnormalities that ultimately lead to malignant transformation and clonal expansion.[2,3] Expansion of the aberrant clone is characterized by morphological dysplasia, impaired differentiation and defective cellular functions, resulting in peripheral cytopenias that frequently involve the erythroid, myeloid, and megakaryocytic lineages.[4] Programmed cell death is often up-regulated in early MDS, due to the enhanced proliferative capacity of MDS clones, and may also contribute to the peripheral cytopenias. Progression to leukemia is usually associated with abrogation of apoptosis.[3] The clinical symptoms resulting from these cellular defects are transfusion-dependent anemia, an increased risk of infection or hemorrhage, and a potential progression to AML.[5]

The myelodysplastic syndromes can be classified into high-risk or low-risk groups according to the French American British (FAB) or the more recently established World Health Organization (WHO) classification systems.[6,7] These classifications are based on the number of blast cells in the bone marrow and peripheral blood, the morphology of cells and cytogenetic abnormalities. Progression towards leukemia is rare in low-risk MDS and life expectancy is relatively long, whereas in the high-risk groups progression rates towards AML are significantly higher.[8]

Cytogenetic abnormalities are common and are observed in about half the cases of primary MDS and in 90% of secondary, therapy-related MDS.[9] The abnormalities observed in MDS are predominantly specific chromosomal deletions, suggesting a pathogenic mechanism based on loss of tumor suppressor genes or of genes necessary for normal hematopoiesis. The most frequently observed cytogenetic abnormalities in MDS include loss of chromosome 7 or partial deletions of chromosome arms 5q, 20q, or 7q.[10-12] While the majority of putative tumor suppressors in MDS remain unknown, several chromosomal translocation-mediated oncogenes and tumor suppressors have been identified. Gene inactivation is responsible for a relatively small number of MDS cases and the genes involved include *p53*, *RB*, *NF1*, *C/EBPα*, and nucleophosmin.[13-19] Activating mutations in the RAS proto-oncogene, FLT3 duplications, loss-of-function point mutations in the gene encoding the AML1/RUNX1 transcription factor and p15 promoter hypermethylation have also been associated with disease progression to AML.[20-23] However, none of these observed alterations is specific for MDS and the underlying molecular causes of the disease remain poorly understood.

Although a number of genetic defects in MDS progenitors have been described, the intracellular signaling pathways underlying deregulation of myelopoiesis have scarcely been investigated thus far. Through identification of the intracellular components responsible for dysfunctional hematopoiesis it will be possible to develop novel treatment strategies for MDS and AML.

To investigate defects in intracellular signaling pathways in MDS CD34$^+$ hematopoietic progenitor cells, we developed an *ex vivo* hematopoiesis culture system. Furthermore, in an attempt to improve the proliferation, survival and differentiation of MDS CD34$^+$ progenitors, cells were treated with the p38MAPK pharmacological inhibitor SB203580, or retrovirally transduced to ectopically express active protein kinase B (PKB/c-akt), or the transcriptional regulators STAT5, C/EBPα or ID1.

Design and Methods

Isolation and culture of human CD34$^+$ cells

Mononuclear cells were isolated from bone marrow of healthy subjects and MDS patients by density centrifugation over a Ficoll-paque solution (density 1.077 g/mL). MACS immunomagnetic cell separation (Miltenyi Biotech, Auburn, CA, USA) using a hapten-conjugated antibody against CD34, which was coupled to beads, was used to isolate CD34$^+$ cells. CD34$^+$ cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Paisley, UK) supplemented with 9% fetal calf serum (FCS) (Hyclone, Logan, UT, USA), 50 μM-mercaptoethanol, 10 U/mL penicillin, 10 μg/mL streptomycin, and 2 mM glutamine at a density of 0.3×10^6 cells/mL. Cells were differentiated towards neutrophils upon addition of stem cell factor (SCF) (50 ng/mL), FLT-3 ligand (50 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 nmol/L), and granulocyte colony-stimulating factor (G-CSF) (30 ng/mL). Every 3 days, cells were counted and fresh medium was added to a density of 0.5×10^6 cells/mL. After 3 days of differentiation, only G-CSF was added to the cells. Pharmacological inhibitors were freshly added to the cells every 3 or 4 days. SB203580 (Alexis Corporation, San Diego, CA, USA) at a dose of 10 μM was used to inhibit p38MAPK activity during granulopoiesis.

Patients

Heparinized human bone marrow cells were collected from MDS patients with a mean age of 62 years (range, 37-78 years) after informed consent had been obtained in accordance with the Declaration of Helsinki. Bone marrow specimens were obtained at diagnosis before treatment. According to the FAB classification, the patients were categorized as having refractory anemia (n=3), refractory anemia with ringed sideroblasts (n=6) or refractory anemia with an excess of blasts (n=1). The patient's characteristics are described in Table 1. None of these patients was treated with G-CSF. Normal bone marrow was obtained from patients undergoing orthopedic surgery who gave informed consent to this collection prior to their operation. The protocols were approved by the human subject review board of the University Medical Center, Groningen.

Histochemical staining of hematopoietic cells

May-Grünwald Giemsa staining was used to analyze myeloid differentiation. Cytospins were prepared from 5×10^4 differentiating granulocytes and were fixed in methanol for 3 min. After fixation cytospins were stained

in a 50% eosin methylene blue solution according to May-Grünwald (Sigma-Aldrich GmbH, Seelze, Germany) for 20 min, rinsed in water for 5 seconds, and the nuclei were counterstained with 10% Giemsa solution (Merck kGaA, Darmstadt, Germany) for 15 min. During neutrophil differentiation, cells could be characterized as differentiating from myeloblast, promyelocyte I, promyelocyte II, myelocyte, and metamyelocytes towards neutrophils with banded or segmented nuclei. These stages can be distinguished by the size of the cells, ratio of cytoplasm versus nucleus present, presence of azurophilic granules and the shape of the nuclei. Differentiated neutrophils were characterized as cells containing either banded or segmented nuclei.

Viral transduction of CD34+ cells

Bicistronic retroviral DNA constructs were utilized, expressing the gene of interest and an internal ribosomal entry site (IRES) followed by the gene encoding for enhanced green fluorescent protein (eGFP) (LZRS-eGFP).[24-26] The retrovirus was produced by stable transfection of the retroviral packaging cell line, Phoenix-ampho by calcium-phosphate co-precipitation. Cells were plated in 6-cm dishes, 24 h before transfection. Ten micrograms of DNA were used per transfection. Medium was refreshed, 16 h after transfection. After an additional 24 h, cells were split into 75-cm^2 culture flasks (Greiner, Frickenhausen, Germany), and 2 μg/mL puromycin was added to the cells. After 2 weeks of selection, cells were grown to a confluence of 90%. Subsequently, cells were grown in a minimal amount of medium for 24 h. Viral supernatants were collected and filtered through a 0.2 μm filter. CD34+ cells were transduced in 24-well dishes precoated with 1.25 μg/cm^2 recombinant human fibronectin fragment CH-296 (RetroNectin; Takara, Otsu, Japan) overnight at 4°C. Transduction was performed by addition of 0.5 mL viral supernatant to 0.5 mL medium containing 0.3×10^6 cells. Twenty-four hours after transduction, 0.7 mL medium was removed from the cells, and 0.5 mL fresh virus supernatant was added together with 0.5 mL fresh medium.

Colony-forming unit assay

Freshly isolated CD34+ cells or retrovirally transduced cells, separated from non-transduced cells by flow cytometry, were used in colony-forming unit (CFU) assays. Cells were plated in IMDM supplemented with 35.3% FCS (Hyclone, Logan, UT, USA), 44.4% methylcellulose-based medium called Methocult (StemCell Technologies, Vancouver, Canada), 11.1 μmol/L of β-mercaptoethanol, 2.2 U/mL of penicillin, 2.2 μg/mL of streptomycin, and 0.44 mmol/L of glutamine at a density of 500 cells/well. CFU assays were done in the presence of SCF (50 ng/mL), FLT-3 ligand (50 ng/mL), GM-CSF (0.1 nmol/L), interleukin-3 (0.1 nmol/L), and G-CSF (0.2 nmol/L). Colonies were scored after 12 days of culture.

Measurement of apoptosis

Apoptotic cells were measured by staining with annexin V (Alexis, Leiden, The Netherlands) according to the manufacturer's protocol. Necrotic cells were visualized in the same assay by staining with propidium iodide.

Western blot analysis

Western blot analysis was performed using standard techniques. In brief, differentiating granulocytes were lysed in Laemmli buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 μg/μL bromophenol blue, and 35 mM β-mercaptoethanol) and boiled for 5 min. Equal amounts of total lysate were analyzed by 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with blocking buffer (Tris buffered saline/Tween-20) containing 5% low-fat milk for 1 h before incubating with an antibody against p38MAPK (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C in the same buffer. Before incubation with antibodies against phosphorylated p38MAPK or phosphorylated MAPKAPK-2 (Cell Signaling Technology, Beverly, MA, USA) for 16 h at 4°C, blots were incubated for 1 h in blocking buffer containing 5% bovine serum albumin (BSA). Blots were subsequently incubated with peroxidase conjugated secondary antibodies for 1 h. Enhanced chemical luminescence (ECL) was used as a detection method according to the manufacturer's protocol (Amersham Pharmacia, Amersham, UK).

Immunohistochemical staining of hematopoietic cells

Cells were first washed in phosphate-buffered saline (PBS) and resuspended in 100 μL 0.5% formaldehyde. After 15 min incubation at 37°C, 900 μL of ice-cold methanol was added to the cells. Cells were washed with PBS after 30 min of incubation on ice and resuspended in PBS/5% FCS (Hyclone, Logan, UT, USA). After 10 min incubation at room temperature, cells were washed and neutrophil progenitors were incubated with an antibody against phosphorylated p38MAPK (Cell Signaling Technology, Beverly, MA, USA) in PBS containing 5% FCS and incubated for another 30 min at 4°C. Cells were washed and subsequently incubated with a phycoerythrin (PE)-conjugated anti-rabbit antibody (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for another 30 min at 4°C. Cells were again washed cells and were analyzed by FACS (FACS Canto II, Beckton Dickinson, Alphen a/d Rijn, The Netherlands).

Statistics

An independent sample *t* test for was performed to compare the differences in proliferation, differentiation, and annexin-positive cells between the controls and cells transduced with STAT5, myrPKB, ID1, or C/EBPα. The same assay was performed to compare cells cultured in either the absence of presence of the pharmacological inhibitor SB203580. A *p* value of 0.05 or less was considered statistically significant.

Results

Myelodysplastic syndrome bone marrow progenitor cells show impaired progenitor expansion and increased levels of apoptosis during neutrophil differentiation

To investigate defects in intracellular signaling pathways in MDS CD34+ hematopoietic progenitor cells, an *ex vivo* differentiation system was utilized as described in

the *Design and Methods* section. Human CD34+ hematopoietic progenitor cells, isolated from the bone marrow of healthy subjects and low-risk MDS patients (Table 1), were cultured in the presence of G-CSF to induce neutrophil differentiation. Cells were cultured for 17 days and differences in expansion, survival and differentiation were analyzed. Expansion of MDS CD34+ cells was dramatically decreased during neutrophil differentiation compared to the expansion of CD34+ cells from healthy controls (Figure 1A). To determine whether this impaired capacity to expand could be due to increased levels of apoptosis, the percentage of annexin-V positive cells was analyzed. The survival of MDS hematopoietic progenitor cells was significantly lower than that of control CD34+ cells during neutrophil development (Figure 1B). These results demonstrate that MDS hematopoietic progenitors have an impaired capacity to proliferate and show decreased survival during neutrophil development *ex vivo*.

Impaired differentiation and decreased colony formation capacity of myelodysplastic syndrome bone marrow progenitor cells during myelopoiesis

To determine whether MDS hematopoietic progenitor cells also had an impaired capacity to differentiate, CD34+ cells were isolated from the bone marrow of healthy subjects and low-risk MDS patients and differentiated towards neutrophils for 17 days. After 14 and 17 days of differentiation, cytospins were prepared to analyze the morphology of the differentiating granulocytes. As expected, MDS bone marrow progenitor cells showed significantly lower percentages of mature neutrophils with banded or segmented nuclei and increased numbers of undifferentiated myeloblasts after 14 and 17 days of neutrophil differentiation (Figure 2A, 2B). In addition, to assess the clonogenic potential of progenitor cells, CD34+ cells from controls and low-risk MDS patients were plated in CFU assays, and colony formation was analyzed after 12 days of culture. Granulocyte-macrophage (GM) colony-forming units (CFU-GM) of MDS CD34+ cells were reduced compared to control CD34+ cells (Figure 2C). Together these results demonstrate that CD34+ bone marrow cells from low-risk MDS patients have an impaired capacity to proliferate as well as to differentiate and show increased levels of apoptosis during neutrophil development. Importantly, this *ex vivo* system is representative of published observations in MDS bone marrow *in vivo*.

Inhibition of p38 MAPK does not improve maturation or increase clonogenic capacity of myelodysplastic hematopoietic progenitor cells

A recent study showed that p38MAPK is constitutively activated in hematopoietic cells from patients with low-risk MDS.[27] Inhibition of p38MAPK activity resulted in decreased apoptosis and stimulated colony formation of primary MDS progenitors, indicating a role for p38MAPK in the pathogenesis of MDS.[27] To determine whether aberrant p38MAPK activation may be involved in the defective neutrophil differentiation observed in MDS primary progenitors, CD34+ hematopoietic progenitor cells isolated from controls and patients with MDS were cultured in the presence of G-CSF to induce neutrophil dif-

Table 1. Patients' characteristics.

Patient n.	Age (years)	MDS type	Hb (g/dL)	Leukocytes (×10⁹/L)	Platelets (×10⁹/L)	Gran. (%)
1	37	RA	Transf.	7.4	30	40
2	77	RARS	5.5	6.4	402	38
3	78	RARS	6.0	5.8	200	40
4	70	RARS	6.0	4.8	297	42
5	60	RARS	Transf.	2.9	153	66
6	58	RARS	Transf.	3.0	173	62
7	50	RAEB	5.7	3.9	93	22
8	68	RA	6.5	6.5	218	30
9	62	RA	6.6	3.9	63	18
10	78	RARS	6.6	4.8	475	25

RA: refractory anemia; RARS: refractory anemia with ring sideroblasts; RAEB: refractory anemia with excess blasts; Transf: transfusion-dependent; Gran: granulocyte percentage in the peripheral blood. None of the patients had cytogenetic abnormalities.

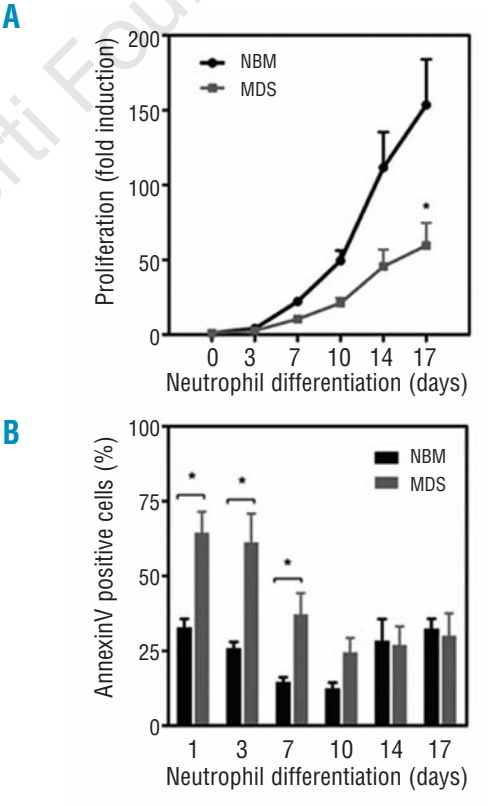

Figure 1. Myelodysplastic syndromes progenitor cells show impaired expansion and increased levels of apoptosis during neutrophil differentiation. CD34+ hematopoietic progenitor cells, isolated from the bone marrow of healthy subjects (NBM) and patients with low-risk myelodysplastic syndromes were cultured for 17 days in the presence of G-CSF to induce neutrophil differentiation. (A) Expansion was determined by counting the trypan blue-negative cells. (B) During the 17-day culture period the percentage apoptotic cells was determined by annexin V staining. Results are presented as means of five independent experiments. Error bars represent SEM.

ferentiation. After 6 days of culture, protein lysates were made and western blot analysis was performed using an antibody against phosphorylated p38MAPK or p38MAPK as a control for equal loading (Figure 3A). In contrast to the previous report, CD34+ cells from low-risk MDS patients did not show elevated levels of phosphorylated p38MAPK compared to controls. In addition, flow cytometric analysis using an antibody against phosphorylated p38MAPK was performed to further support these observations (Figure 3B).

In order to investigate whether inhibition of p38MAPK can indeed improve final maturation of MDS progenitors during myelopoiesis, CD34+ cells isolated from patients with MDS were differentiated towards neutrophils in either the presence or absence of the specific p38MAPK inhibitor SB203580. The efficacy of SB203580 was confirmed by its ability to inhibit the phosphorylation of MAPKAPK-2 (MK2) in CD34+ cells, a direct target of p38MAPK (Figure 3C). After 17 days of differentiation, cytospins were prepared to analyze the morphology of the differentiating granulocytes. Inhibition of p38MAPK activity did not improve neutrophil development of

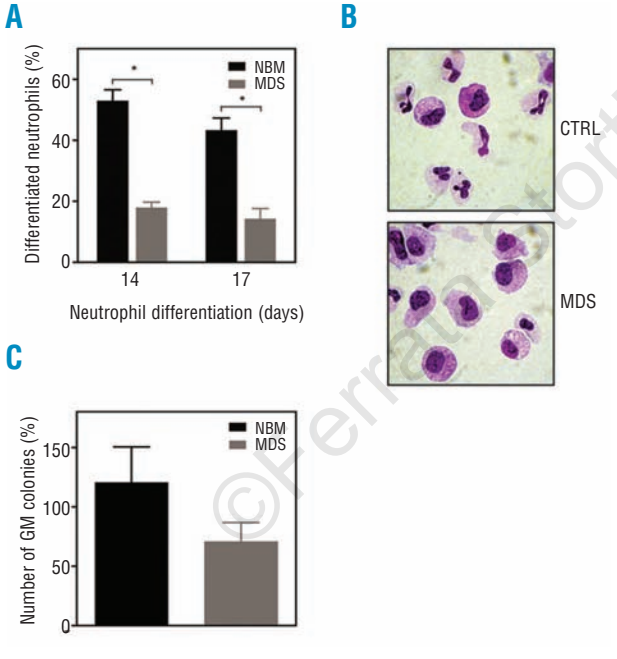

Figure 2. Impaired differentiation and decreased GM colony formation capacity of myelodysplastic syndromes (MDS) CD34+ progenitors during myelopoiesis. CD34+ hematopoietic progenitor cells, isolated from the bone marrow of healthy subjects (NBM) and patients with low-risk MDS were cultured for 17 days in the presence of G-CSF to induce neutrophil differentiation. After 14 and 17 days of differentiation, (B) cytospins were prepared and stained with May-Grünwald Giemsa solution. (A) Data are expressed as the percentage of differentiated neutrophils. (C) CD34+ progenitor cells from controls and patients with low-risk MDS were plated in CFU assays, and colony formation was analyzed after 12 days. Results in Figures 2A and 2C are presented as means of six different MDS samples (2 with refractory anemia, 3 with refractory anemia with ringed sideroblasts, 1 with refractory anemia with excess blasts), of which four are also included in the experiments described in Figure 3 (2 with refractory anemia, 2 with refractory anemia and ringed sideroblasts). Error bars represent SEM.

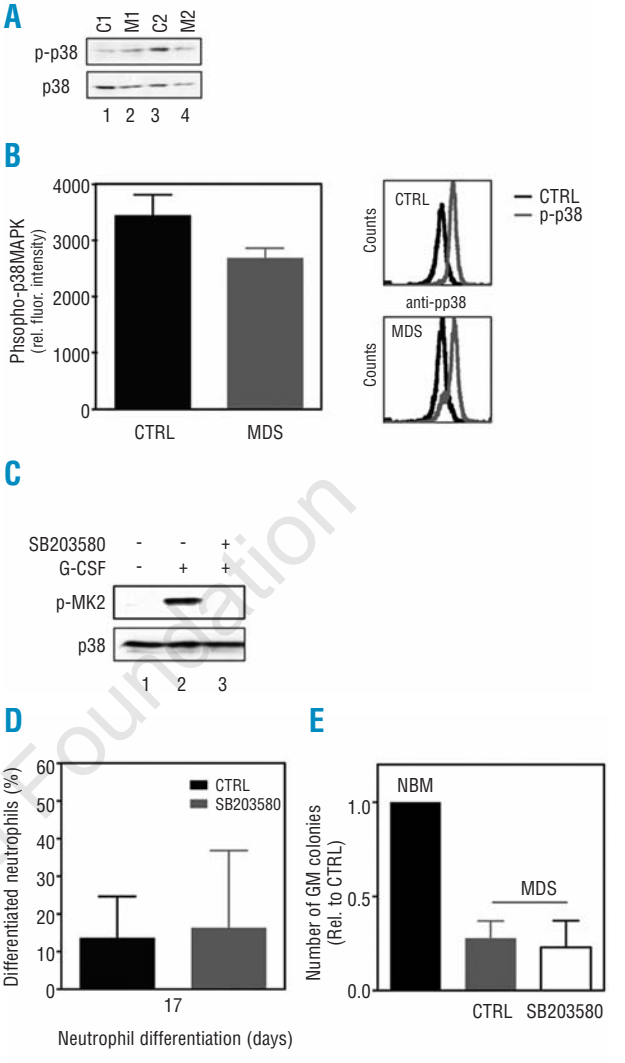

Figure 3. Inhibition of p38MAPK does not improve maturation or increase clonogenic capacity of myelodysplastic syndromes (MDS) progenitor cells. (A) CD34+ cells, isolated from bone marrow of healthy subjects (lanes 1 and 3) and patients with low-risk MDS (lanes 2 and 4), were cultured in the presence of G-CSF to induce neutrophil differentiation. After 6 days of culture, protein lysates were made and western blot analysis was performed using an antibody against phosphorylated p38MAPK, or p38MAPK as a control for equal loading. (B) Activation of p38MAPK was further analyzed by flow cytometry using an antibody against phosphorylated p38MAPK. (C) CD34+ cells were cultured in the presence of G-CSF to induce neutrophil differentiation. After 6 days of culture, cells were left untreated (lanes 1 and 2) or treated with SB230580 (lane 3) for 45 min before stimulation with G-CSF (lanes 2 and 3) for 15 min. Protein lysates were prepared and western blot analysis was performed with an antibody against phosphorylated MAPKAPK2 or p38MAPK as a control for equal loading. (D) After 17 days of differentiation, cytospins were prepared to analyze the morphology of the differentiating granulocytes. Data are expressed as the percentage of differentiated neutrophils. (E) CD34+ progenitor cells isolated from patients with low-risk MDS were plated in CFU assays in either the presence or absence of 10 μM SB203580 and colony formation was analyzed after 12 days. Results are presented as means of three independent experiments. Error bars represent SEM.

MDS CD34+ cells (Figure 3D). In addition, to determine whether inhibition of p38MAPK can improve the clonogenic capacity of primary MDS progenitor cells, CD34+ cells from low-risk MDS patients were plated in CFU assays, and colony formation was analyzed after 12

days. Treatment of MDS hematopoietic progenitor cells with SB203580 did not result in significantly increased CFU-GM (Figure 3E). Together these results suggest that inhibition of p38MAPK is not sufficient to restore final maturation or improve the clonogenic capacity of low-risk MDS hematopoietic progenitor cells.

Increased PKB or STAT5a activity is not sufficient to restore neutrophil development in myelodysplasia

Hematopoietic cytokines can activate several signal transduction pathways, which have been shown to be involved in the regulation of myeloid differentiation, including p38MAPK, phosphatidylinositol 3 kinase (PI3K), and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway.[28] Recently we have demonstrated that during myelopoiesis PKB activity is essential for both hematopoietic progenitor survival and neutrophil development.[26] We also observed that CD34$^+$ cells from low-risk MDS patients show decreased PKB activation in response to the chemo-attractant SDF-1.[29] Furthermore, neutrophils isolated from low-risk MDS patients also exhibited decreased PKB phosphorylation upon stimulation with fMLP.[30] However, CD34$^+$-derived neutrophil progenitor cells from low-risk MDS patients did not show significantly altered levels of phosphorylated PKB compared to corresponding cells from controls (*Online Supplementary Figure S1*). To further determine whether aberrant PKB activation may be involved in the defective neutrophil differentiation observed in MDS primary progenitors, we used of a bicistronic retroviral DNA construct co-expressing eGFP and a constitutively active form of PKBα (myrPKB). A retrovirus was generated and used to transduce low-risk MDS CD34$^+$ cells, which were cultured in the presence of G-CSF to induce neutrophil differentiation. Three days after transduction, eGFP-positive cells were sorted by FACS from the non-transduced cells. After 14 and 17 days of differentiation, cytospins were prepared and the morphology of the cells was analyzed after May-Grünwald Giemsa staining. Activation of PKB did not improve the reduced neutrophil development from hematopoietic progenitors isolated from patients with MDS (Figure 4A), indicating that activation of PKB alone is not sufficient to rescue neutrophil development in MDS.

We and others have also shown that activation of the transcription factor STAT5 regulates proliferation, apoptosis and differentiation during erythroid and myeloid development.[24,31] To address the question of whether STAT5 signaling plays a critical role in regulating neutrophil differentiation in MDS, a bicistronic retroviral DNA construct co-expressing STAT5a and eGFP was used to transduce CD34$^+$ cells isolated from MDS patients. After 17 days of differentiation, eGFP positive cells were sorted by FACS from the non-transduced cells and cytospins were prepared. Ectopic expression of STAT5a resulted in a modest increase in the percentage of mature neutrophils with banded or segmented nuclei compared to cells transduced with eGFP alone (Figure 4B). Together these data indicate that despite playing essential roles in granulopoiesis, ectopic expression of myrPKB or STAT5a alone is not sufficient to restore neutrophil differentiation in MDS.

Increased C/EBPα and ID1 expression results in improved neutrophil production in patients with low-risk myelodysplastic syndromes

One of the key transcriptional regulators involved in lineage choice decisions during myeloid differentiation is the CCAATT/enhancer binding protein α (C/EBPα). Genetic alterations and reduced expression of the C/EBPα gene have been found in both AML and MDS, which supports the involvement of deregulated C/EBPα expression in the inefficient granulopoiesis characteristic of MDS.[18,32-34] In addition, another transcriptional regulator that has been demonstrated to play an important role in the regulation of proliferation and differentiation during myelopoiesis is inhibitor of DNA binding protein 1 (ID1).[25] To investigate whether ectopic expression of ID1 or C/EBPα can indeed improve myeloid maturation of MDS progenitors, CD34$^+$ hematopoietic progenitor cells, isolated from patients with MDS, were transduced with ID1, C/EBPα or eGFP as a control and were cultured in the presence of G-CSF to induce neutrophil differentia-

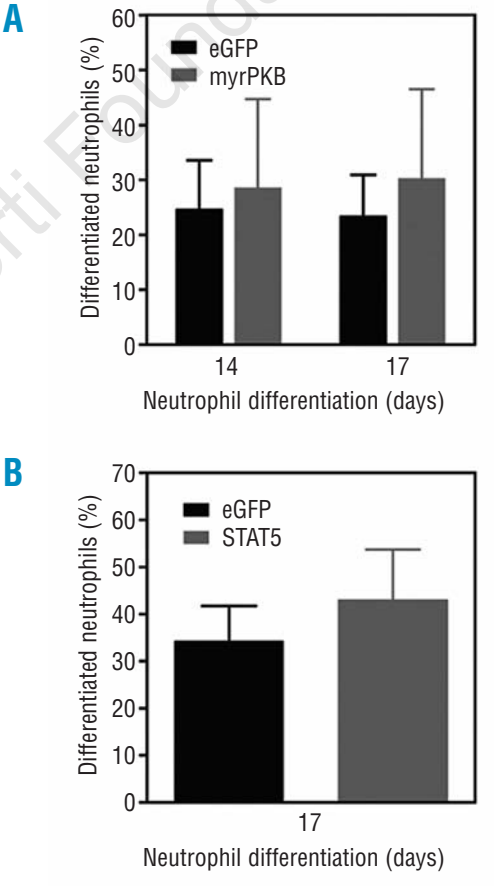

Figure 4. Increased PKB or STAT5a activity is not sufficient to restore neutrophil development in myelodysplastic syndromes. CD34$^+$ cells, isolated from patients with low-risk myelodysplastic syndromes were retrovirally transduced with (A) myrPKB, (B) STAT5a or eGFP as a control and cultured in the presence of G-CSF to induce neutrophil differentiation. After 17 days of culture, transduced cells were separated from non-transduced cells by FACS, and cytospins were prepared. Data are expressed as the percentage of differentiated neutrophils. Results are presented as means of four independent experiments. Error bars represent SEM.

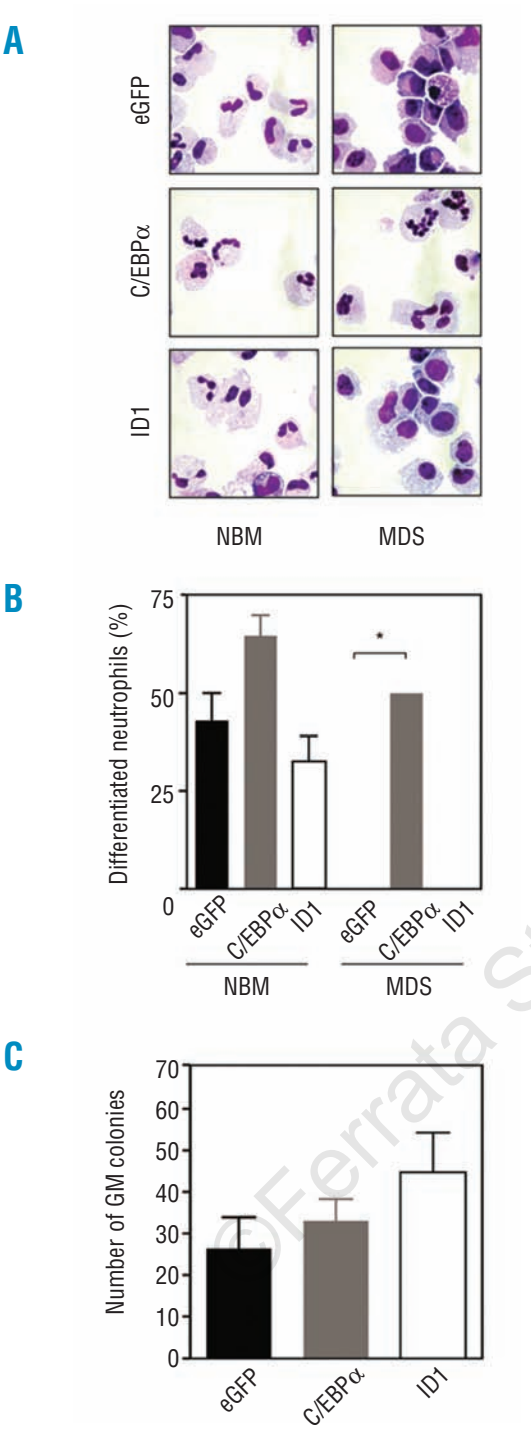

Figure 5. Ectopic expression of C/EBPα and ID1 results in improved neutrophil production in low-risk myelodysplastic syndromes (MDS). (A) CD34⁺ progenitor cells, isolated from the bone marrow of healthy subjects or patients with low-risk MDS were retrovirally transduced with C/EBPα, ID1 or eGFP alone and cultured in the presence of G-CSF to induce neutrophil differentiation. Subsequently, transduced cells were separated from non-transduced cells by FACS and after 17 days of culture, cytospins were prepared. (B) Data are expressed as the percentage of differentiated neutrophils. (C) CD34⁺ cells isolated from MDS patients were retrovirally transduced with C/EBPα, ID1 or eGFP alone. After 3 days, transduced cells were separated from non-transduced cells by FACS, plated in CFU assays and colony formation was analyzed after 12 days. Results are presented as means of three independent experiments. Error bars represent SEM.

tion. Three days after transduction, eGFP positive cells were sorted by FACS from the non-transduced cells. After 17 days of differentiation, cytospins were prepared and the morphology of the cells was subsequently analyzed after May-Grünwald Giemsa staining (Figure 5A).

Ectopic expression of ID1 did not improve the reduced neutrophil development of MDS CD34⁺ cells. However, transduction of MDS CD34⁺ cells with C/EBPα resulted in a dramatic increase in the percentage of neutrophils with banded or segmented nuclei (Figure 5B). These results demonstrate that ectopic expression of C/EBPα is sufficient to restore neutrophil development of MDS hematopoietic progenitors.

In addition, to determine whether ID1 or C/EBPα can improve the clonogenic capacity of MDS CD34⁺ cells, CFU assays were performed, and colony formation was analyzed after 12 days of culture. Interestingly, ectopic expression of both ID1 and C/EBPα resulted in increased GM colony formation (Figure 5C). Together these data suggest that targeting the ID1 and C/EBPα transcriptional regulators may be of benefit in the design of novel therapies for low-risk MDS.

Discussion

Although MDS are some of the most prevalent hematologic disorders, the defects in the intracellular signaling pathways responsible for aberrant hematopoiesis in these conditions remain largely undefined. In the present study, we investigated whether known regulators of myeloid differentiation can improve neutrophil development of MDS CD34⁺ hematopoietic progenitor cells utilizing a human *ex vivo* granulocyte differentiation system. Our data demonstrate that ID1 and C/EBPα both play a role in expansion and differentiation during myeloid development of MDS hematopoietic progenitors. Ectopic expression of ID1 and C/EBPα resulted in enhanced GM colony formation, whereas treatment of progenitors with SB203580, PKB or STAT5 did not improve colony formation. In addition, C/EBPα-transduced MDS progenitors exhibited greatly improved neutrophil maturation.

Using an *ex vivo* differentiation system to study the defects in intracellular signaling pathways in MDS CD34⁺ hematopoietic progenitor cells, we were able to demonstrate that CD34⁺ bone marrow cells from low-risk MDS patients have both an impaired capacity to proliferate and show increased levels of apoptosis during neutrophil development. Importantly, this *ex vivo* system thus mimics the published observations in MDS bone marrow *in vivo*.[35]

p38MAPK is a serine-threonine kinase, originally discovered as a stress-activated kinase, which has been demonstrated to be involved in the regulation of differentiation of various cell types, including granulocytes, with its effects being cell type- and context-specific.[36] Inhibition of p38MAPK activity was shown to enhance neutrophil development, while constitutive activation of the MKK3/p38MAPK signaling module dramatically inhibited neutrophil differentiation (*unpublished data*). In addition, it was recently shown that p38MAPK is consti-

tutively activated in the bone marrow of patients with low-risk MDS. Inhibition of p38MAPK activity was found to decrease apoptosis and stimulate GM colony formation in primary MDS progenitors, suggesting a role for p38MAPK in the pathogenesis of this syndrome.[37,27] However, our data indicate that inhibition of p38MAPK is not sufficient to restore final maturation or improve the clonogenic capacity of low-risk MDS hematopoietic progenitors (Figure 3D, 3E). Moreover, CD34$^+$ cells from low-risk MDS patients did not show elevated levels of phosphorylated p38MAPK compared to control cells (Figure 3A, 3B). These differences might be explained by donor variations between MDS patients' samples as MDS comprise a heterogeneous group of stem cell disorders. In addition, although Navas *et al.* demonstrated that inhibition of p38MAPK stimulates GM colony formation in primary low-risk MDS progenitors,[27] they did not characterize the morphology of the differentiating neutrophils.

PI3K has been demonstrated to play a critical role in the survival and proliferation of a variety of cell types and recent evidence showed that PI3K and its downstream effector PKB also play an important role in regulating hematopoiesis.[26] Previously, we demonstrated that CD34$^+$ cells from low-risk MDS patients show decreased PKB phosphorylation in response to the chemo-attractant SDF-1.[29] In addition, constitutive activation of PKB in bone marrow mononuclear cells from patients with high-risk MDS was reported, while mononuclear cells from normal bone marrow and patients with low-risk MDS demonstrated low levels or no PKB activation.[38] Taken together, these findings suggest that aberrant PKB activation might be one of the factors contributing to the ineffective hematopoiesis observed in MDS. However, activation of PKB in MDS CD34$^+$ hematopoietic progenitor cells did not improve aberrant neutrophil development, indicating that activation of PKB alone is also insufficient to rescue neutrophil development in low-risk MDS (Figure 4A).

Several studies suggest that STAT5 may play a critical role in neutrophil development. Loss of STAT5 function in primary bone marrow cells, for example, leads to a reduction in CFU-G colony formation, while bone marrow cells from mice lacking STAT5 are unable to repopulate the myeloid lineage of lethally irradiated wild-type recipient mice.[39-42] Furthermore, it has been demonstrated that STAT5 favors the survival of myeloid progenitors by inducing expression of the anti-apoptotic protein Bcl-xL.[43] However, although STAT5 expression has been shown to be essential during myelopoiesis, our data indicate that expression of STAT5a is again not sufficient to restore neutrophil development in low-risk MDS (Figure 4B).

ID proteins function as inhibitors of members of the basic helix-loop-helix family of transcription factors and have been demonstrated to play an important role in regulating proliferation and differentiation of a variety of cell lineages.[44] It has been shown that ID1 mRNA levels are often high in proliferating cells, but are down-regulated in differentiating cells.[45] We have previously shown that ID1 levels are upregulated during early granulopoiesis, then decrease during final maturation.[25] Our data suggest that ectopic expression of ID1 is not sufficient to improve neutrophil differentiation in MDS; however, GM colony formation of MDS hematopoietic progenitors was significantly increased, suggesting that ID1 may exert its major effects on progenitor expansion during the early phase of granulopoiesis. Previous studies have demonstrated that aberrant activation of ID proteins can contribute to tumorigenesis by stimulating proliferation and facilitating neovascularization. In addition, analysis of various solid and leukemic human tumors have revealed that the level of expression of ID proteins is often elevated.[46-49] While this suggests that targeting ID1 may be of benefit in the design of novel therapies for low-risk MDS, manipulation for therapeutic purposes will not be without risk.

C/EBPα is a leucine zipper transcription factor that plays a critical role in normal myelopoiesis. Expression of C/EBPα is detectable in early myeloid precursors and is upregulated upon commitment to granulocytes.[50,51] Consistent with this expression pattern, mice deficient in C/EBPα lack mature neutrophils and accumulate immature myeloblasts in the bone marrow.[52] Conversely, ectopic expression of C/EBPα in precursor cell lines triggers neutrophil differentiation.[53,54] Mutations within the C/EBPα gene are found in approximately 9% of patients with AML, leading to production of C/EBPα mutants deficient in DNA binding.[32,33,34] C/EBPα levels are also affected by various leukemic fusion proteins through mechanisms that involve transcriptional as well as translational repression.[55-57] Although, alterations in the C/EBPα gene have been found in patients with AML, they seem to be less frequently observed in MDS patients.[17] However, in patients with 5q- syndrome, a distinct clinical subgroup of MDS, the gene encoding C/EBPα was found to be extensively down-regulated in MDS progenitor cells.[18] Besides mutations in the CEBPα gene itself, C/EBPα transcription may be repressed by DNA promoter hypermethylation. Methylation of DNA is a commmon epigenetic modification, which plays an important role in correct regulation of gene expression in mammalian cells. Hypermethylation of promoter residues and consequent inactivation of regulatory genes has been found to play a pathogenetic role in the development of MDS.[58] Recent data have shown that in a specific subgroup of AML, which phenotypically resembles AML with mutations in C/EBPα, the CEBPα gene was silenced due to promoter hypermethylation.[59] Our results demonstrate that ectopic expression of C/EBPα is sufficient to restore neutrophil development of MDS hematopoietic progenitors, supporting the hypothesis that abrogation of granulopoiesis in MDS patients is, at least in part, due to aberrant C/EBPα expression or functionality in the bone marrow. Interestingly, the major effect of C/EBPα was observed on neutrophil differentiation rather than on CFU-GM growth, arguing for a key role in granulocytic differentiation. Consistently, previous studies showing that expression of C/EBPα is detectable in early myeloid precursors and is upregulated upon commitment to granulocytes, indicate that C/EBPα may indeed exert its major effects on progenitor maturation during the late phase of granulopoiesis.[50,51]

G-CSF is an essential cytokine for both the prolifera-

tion of myeloid precursors and their differentiation into mature neutrophils. It is tempting to speculate that the number of G-CSF receptors expressed on the membrane of progenitors may play a critical role in the maturation defect in myelodysplastic patients. Previous studies demonstrated that CEBPα plays an important role in transcriptionally regulating G-CSF receptor expression, by direct interaction with the G-CSSFR promoter.[60,61] Moreover, decreased G-CSF receptor expression on $CD34^+$ cells was found in a significant proportion of patients with both low-risk and high-risk MDS.[62] Interestingly, MDS patients with low receptor expression had a strong predisposition to develop neutropenia and a poor or absent response to G-CSF administration. It could, therefore, be hypothesized that increased expression of C/EBPα in low-risk MDS $CD34^+$ cells may result in enhanced G-CSF receptor expression, leading to more efficient signaling in response to G-CSF, ultimately resulting in improved neutrophil development.

In conclusion, while a variety of genetic alterations have been reported to be involved in the pathogenesis of MDS, our data suggest that targeting C/EBPα may be sufficient in the design of novel therapies for low-risk MDS.

Authorship and Disclosures

CRG designed the research, performed experiments, made the figures, analyzed results, and wrote the paper; MB designed the research, performed experiments, made the figures, analyzed results, and wrote the paper; EV designed the research and analyzed results; PJC designed the research, analyzed results and wrote the paper.

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