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PhD THESIS

The possible involvement of mesenchymal stem cells (MSCs) in the pathophysiology of bone marrow (BM) failure associated with myelodysplastic syndromes (MDS)

Presented by

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Summary

Defective hematopoiesis supporting capacity of bone marrow (BM) stroma has been implicated in the pathophysiology of myelodysplastic syndromes (MDS).

The aim of this study is to explore whether the BM stroma progenitors, namely the mesenchymal stem cells (MSCs), are primarily affected in MDS by evaluating the reserves, the functional properties, as well as the cytogenetic characteristics, in comparison to BM hematopoietic cells, in patients with de novo MDS (*n* = 13). The number, differentiation potential toward adipocytes/chondrocytes/osteoblasts and immunosuppressive function in terms of inhibition of mitogen-induced T-cell proliferation did not differ significantly between patient and normal (*n* = 20) MSCs. Patient MSCs did not show any aberrations in the production of proinflammatory or growth-promoting cytokines and did not harbour the cytogenetic abnormalities present in hematopoietic cells. Occasional patient and normal MSC cultures, however, developed irrelevant chromosomal alterations (trisomies 5 and 7) with uncertain pathophysiologic significance. Compared to controls, patient MSCs displayed impaired proliferative and clonogenic potential through passages that might represent a nonspecific abnormality associated with the chronic inflammatory process present in patients' BM.

These data suggest that BM MSCs from MDS patients do not belong to the abnormal clone and do not represent the main cellular source contributing to the inflammatory marrow microenvironment.

Περίληψη

Εισαγωγή: Διαταραχές του αιμοποιητικού μικροπεριβάλλοντος εμπλέκονται στην παθοφυσιολογία των Μυελοδυσπλαστικών Συνδρόμων (ΜΔΣ).

Σκοπός της παρούσας εργασίας είναι η διερεύνηση της πιθανότητας ύπαρξης πρωτοπαθών διαταραχών στα μυελικά μεσεγχυματικά προγονικά κύτταρα (ΜΠΚ) ασθενών με ΜΔΣ.

Ασθενείς και Μέθοδοι: Μελετήθηκαν 13 ασθενείς με πρωτοπαθές ΜΔΣ και 20 υγιείς μάρτυρες. Ειδικότερα, μελετήσαμε τον αριθμό, τα κυτταρογενετικά και λειτουργικά χαρακτηριστικά των μυελικών ΜΠΚ και πιο συγκεκριμένα, την ικανότητα πολλαπλασιασμού, διαφοροποίησης και καταστολής της διεγερσιμότητας των Τ-λεμφοκυττάρων *in vitro*.

Αποτελέσματα: Ο αριθμός των ΜΠΚ στο κλάσμα των μυελικών μονοπυρήνων κυττάρων, η ικανότητα διαφοροποίησης προς λιποκύτταρα, χονδροκύτταρα και οστεοκύτταρα αλλά και η ανοσοκατασταλτική τους δράση σε επαγώμενα από μιτογόνα Τ-λεμφοκύτταρα, δεν διέφερε σημαντικά μεταξύ ασθενών και μαρτύρων. Τα επίπεδα προφλεγμονωδών κυτταροκινών και αυξητικών παραγόντων στα υπερκείμενα καλλιεργειών ΜΠΚ δεν διέφεραν, επίσης, μεταξύ ασθενών και μαρτύρων. Τα ΜΠΚ ασθενών με παθολογικό καρυότυπο δεν εμφάνισαν τις κυτταρογενετικές ανωμαλίες των αντίστοιχων αιμοποιητικών κυττάρων. Ωστόσο, κάποιες από τις καλλιέργειες ΜΠΚ εμφάνισαν μη αναμενόμενες κυτταρογενετικές ανωμαλίες (τρισωμία 5 και 7) τόσο στους ασθενείς όσο και στους μάρτυρες, γεγονός με άγνωστη παθοφυσιολογική σημασία. Τα ΜΠΚ των ασθενών είχαν μειωμένη πολλαπλασιαστική και κλωνογονική ικανότητα σε όλη τη διάρκεια των ανακαλλιεργειών, εύρημα, πιθανότατα μη-ειδικό, οφειλόμενο στην μακροχρόνια επίδραση του φλεγμονώδους περιβάλλοντος.

Συμπέρασμα: Τα δεδομένα της μελέτης αποδεικνύουν ότι τα ΜΠΚ των ασθενών με ΜΔΣ δεν ανήκουν στον παθολογικό κλώνο και δεν αποτελούν την κύρια κυτταρική πηγή του φλεγμονώδους μυελικού μικροπεριβάλλοντος.

1. Introduction

1.1 Myelodysplastic Syndromes (MDS)

1.1.1 MDS definition

Myelodysplastic syndromes (MDS) represent a heterogeneous group of acquired clonal myeloid stem cell disorders characterized by ineffective peripheral blood cell production with Mono-, Bi- or Pancytopenia (megakaryocytic, erythroid and/or granulocytic/monocytic lineages), bone marrow (BM) failure (most commonly a hypercellular, dysplastic-appearing bone marrow), and a genetic instability with enhanced risk of disease progression into acute myeloid leukemia (AML) ([1], [2], [3]).

The risk to develop a MDS increases with age as the disease most commonly affects individuals between the ages 58 to 75 years, median age 60 years. The incidence amounts 4-5/100000/year, and at the age of 70 years 20-50/100000/year. With exception of the 5q- syndrome (specially classified in the MDS group, see below), where females are affected more often than males (male/female=3/7), there is no predominance in gender ([4]).

Concerning the aetiology, primary (*de novo*) MDS are separated from secondary MDS. More than 90% are *de novo* MDS, by definition without any knowledge about an initiating event. Secondary MDS can be observed after chemotherapy for other neoplasms (therapy-associated MDS, t-MDS), high dose chemotherapy/stem cell transplantation and/or radiotherapy ([5]). Secondary MDS are often more severe and more difficult to treat than *de novo* MDS ([6], [7]).

The symptoms of MDS depend on the disease stage. In about 50% a MDS is diagnosed in a routine check up without any symptoms. Most of the symptoms associated with MDS are caused by peripheral cytopenia of red blood cells (paleness, weakness, short of breath), white blood cells (increased incidence of

infections) and/or platelets (bleeding, hematoma) ([8]). In the last years it has become more important to evaluate the MDS type as there are individual therapy strategies depend on MDS subtype and a risk score system ([9]).

1.1.2 MDS classification

On the base of morphology, cytochemistry, immunophenotype, and cytogenetic characteristics of peripheral blood and bone marrow cells a MDS classification (and separation from AML cases) is made.

Until 1999, a classification system developed in the 1970s by a French-American-British (FAB) Co-operative Group (FAB classification) was used for MDS classification ([10]). This classification system was worldwide generally accepted, and may be still sometimes used as it was in use for so long time, and survival curves of MDS patients treatment may still use the FAB categories: RA (Refractory anemia), RARS (Refractory anemia with ringed sideroblasts), RAEB (Refractory anemia with excess blasts), RAEB-T (Refractory anemia with excess blasts in transformation), and CMML (Chronic myelomoncytic leukemia).

However, the FAB classification is missing a detailed characterization for morphologic abnormalities so far not further subdivided such as the 5q- syndrome or realization that blast counts in BM exceeding 20% usually show a prognosis similar to AML.

The 5q- syndrome has been first described by Van den Berghe in 1974 ([11]). It is clinically characterized by an indolent course and female preponderance as well as refractory macrocytic anaemia, normal or elevated platelets and mild leukopenia. The bone marrow smears reveal <5% blasts and as an especially distinct feature show hypolobulated megakaryocytes. However, about 90% of MDS with del(5q) do not have a 5q- syndrome. So that del(5q) in these cases can be just a component of the 5q- syndrome ([12], [13]).

Further modifications of the MDS classification were suggested and a modified classification was proposed by the World Health Organization (WHO). The new WHO classification recognizes unilineage dysplasia for a diagnosis of RA with and without sideroblasts, the 5q– syndrome was redefined as a specific type of MDS (isolated del 5q but restricting the entity to cases without excess of marrow blasts

(i.e., < 5% blasts)), redefines AML with more than 20% blasts in blood and BM, and CMML was removed from the MDS and put in a new category of myelodysplastic-myeloproliferative overlap syndromes. Thus, the WHO (World Health Organisation) system is now the most current MDS classification system and subdivides MDS into 8 groups (table 1).

Table 1. The WHO 2008 classification of MDS (Swerdlow SH, Campo E, Harris NL et al. WHO Classification of Tumours of Hematopoietic and Lymphoid Tissues. Lyon: IARC Press, 2008)

Disease	Blood findings	Bone marrow findings
Refractory cytopenias with unilineage dysplasia (RCUD) Refractory anaemia (RA) Refractory neutropenia (RN) Refractory thrombocytopenia (RT)	Unicytopenia or bicytopenia[a] No or rare blasts (<1%)	Unilineage dysplasia; ≥10% of the cells of the affected lineage are dysplastic <5% blasts <15% of the erythroid precursors are ring sideroblasts
Refractory anaemia with ring sideroblasts (RARS)	Anaemia No blasts	Erythroid dysplasia only <5% blasts ≥15% ringed sideroblasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s) No or rare blasts (<1%) No Auer rods $<1 \times 10^9\ L^{-1}$ monocytes	Dysplasia in ≥10% of cells in two or more myeloid lineages <5% blasts No Auer rods ±15% ring sideroblasts
Refractory anaemia with excess blasts-1 (RAEB-1)	Cytopenias <5% blasts No Auer rods $<1 \times 10^9\ L^{-1}$ monocytes	Unilineage or multilineage dysplasia 5–9% blasts No Auer rods
Refractory anaemia with excess blasts-2 (RAEB-2)	Cytopenias 5–19% blasts ±Auer rods $<1 \times 10^9\ L^{-1}$ monocytes	Unilineage or multilineage dysplasia 10–19% blasts ±Auer rods[b]
Myelodysplastic syndrome, unclassified (MDS-U)	Cytopenias No or rare blasts (<1%) No Auer rods	Unequivocal dysplasia in <10% of cells in one or more myeloid cell lines <5% blasts
MDS associated with isolated del(5q)	Anaemia No or rare blasts (<1%) Platelet count usually normal or increased	Normal to increased megakaryocytes with hypolobated nuclei <5% blasts Isolated del(5q) cytogenetic abnormality No Auer rods

[a]Bicytopenia may occasionally be observed. Cases with pancytopenia should be classified as MDS-U. [b]If the diagnostic criteria for MDS are fulfilled and Auer rods are present, the patient should always be categorized as RAEB-2.

In untreated MDS classified according to the FAB classification, the median survival is about 28 months for patients with RA and 11 months for patients with RAEB, and more than 50% of patients with RARS survive for 3 years (figure 1).

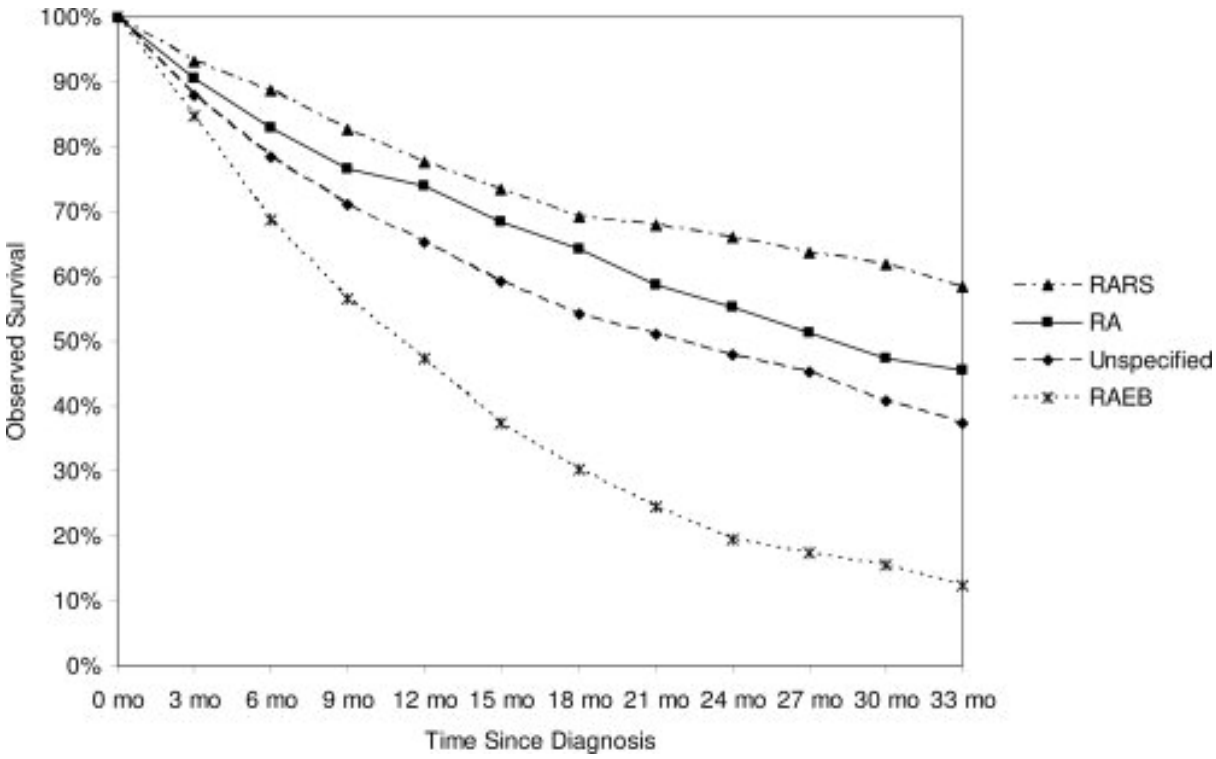


Figure 1. Observed survival of patients with myelodysplastic syndrome (MDS) by subtype in the United States (November 2005). RA indicates refractory anemia; RARS, RA with rare sideroblasts; RAEB, RA with excess blasts ([4]).

1.1.3 MDS cytogenetics

On the cytogenetic level, MDS are characterized by acquired chromosomal abnormalities. These clonal abnormalities are non-random, may give information about aetiology, and have a big impact on patient prognosis and therapy. In *de novo* MDS about 30-55% and in about 80% of secondary MDS cytogenetic aberration are observed ([14]). The most common karyotype abnormalities are: del(5q): 29%, del(7q)/-7: 24%, +8: 16%, del (20q): 7%, -Y: 5%, and complex aberrant karyotypes: 27% (defined as more than 3 unbalanced cytogenetic abnormalities) ([15]). None of these aberrations is specific for MDS as they are also described in AML subtypes. Otherwise, the AML associated abnormalities such as t(15;17)/PML-RARα, t(8;21)/AML1-ETO and inv(16)/CBFb-MYH11 are exclusively found in AML ([16]).

The incidence of karyotype aberrations varies between the MDS subtypes, most frequently observed in RAEB-I and RAEB II, preferential monosomy 7 and complex aberrant karyotypes ([17]).

Interstitial deletions (two breakpoints within one chromosome arm and reunion) of the long arm of chromosome 5, del(5q), are the most common chromosomal abnormalities in patients with MDS. As del(5q) is detected in about 30% of patients with MDS, it is in 14% an isolated anomaly, in 5% as del(5q) with one additional anomaly, and in 11% as part of a more complex karyotype ([17]). Concerning the prognosis, the existence of additional cytogenetic abnormalities is unfavourable demonstrated by median survival and risk for progression into AML (table 2).

Table 2. Progression to acute myeloid leukemia (AML) and survival in main published series of MDS with del 5q ([18])

	No. of Cases	Karyotype	% Marrow Blasts	Median Survival (months)	AML Transformation
Van den Berghe et al[1,2]	43	Isolated del 5q	< 5	28	21%
	23	Del 5q and other	< 5	11	8%
	15	Isolated del 5q	≥ 5	28+	14%
	21	Del 5q and other	≥ 5	4	55%
Mathew et al[5]	43	Isolated del 5q	< 5 or ≥ 5	63	16%
Greenberg et al[7]	48	Isolated del 5q	< 5 or ≥ 5	25	20%
Giagounidis et al[8,9]	53	Isolated del 5q	< 5	107	9%
	9	del 5q and 1 additional rearrangement	< 5	47	
	13	Isolated del 5q	≥ 5	24	80%
	25	del 5q and ≥ 2 additional rearrangements	≥ 5	7.4	

1.1.4 MDS prognosis and therapy

Nowadays, an International Prognostic Scoring System (IPSS) is used for predicting a patients long-term outcome ([19]) (table 3). Considering blast percentage in BM, 0 and/or 3 line cytopenia, and cytogenetics, the MDS is classified into four prognostic risk groups (low risk, intermediate risk-1, intermediate risk-2, and high risk), and

allows a prognosis in untreated MDS patients for overall survival and their risk to develop a secondary leukaemia. The IPSS score together with other clinical patient characteristics like patients age usually are crucial parameters for developing an individual risk adapted therapy strategy.

Table 3. The International Prognostic Scoring System (IPSS) for MDS ([19])

Prognostic variable	**0 points**	**0,5 points**	**1 point**	**1,5 points**
Bone marrow blasts(%)	< 5	5-10		11-20
Karyotype	favourable (normal karyotype, -Y, del(5q), del(20q))	intermediate (all other karyotype abnormalities)	unfavourable (complex </=3 abnormalities); del(7q)/-7	
Number of cytopenia	0-1	2-3		

Risk group	**Total score**	**Median Survival (years)**	**25% AML Evolution (years)**
Low	0	5.7	9.4
Intermediate-1	0.5–1.0	3.5	3.3
Intermediate-2	1.5–2.0	1.2	1.1
High	</=2.5	0.4	0.2

The best treatment for MDS depends on MDS subtype, risk level, age, and overall health. The treatment options include ([9]):

- supportive care
- chemotherapy
- stem cell transplantation
- newer drug therapies

The only curative therapy for patients with MDS is stem cell transplantation (SCT) and may be the treatment of choice for intermediate risk-2/high risk MDS <60 years or MDS in progression if a matched donor is available. Another treatment option for

intermediate risk-2/high risk MDS in good overall health without a suitable donor may be polychemotherapy as it is used for AML. But relapse is common (50%) and the rate of long-term survival is low, particularly in older patients. As the MDS patients are generally older and are unable to tolerate intensive chemotherapy, most of the times the treatment in low risk/intermediate risk-1 MDS is restricted to supportive measures to moderate symptoms caused by the BM insufficiency (blood transfusion, blood cell growth factors (G/GM-CSF, Erythropoietin), antibiotics/antimycotics). Another option for low risk MDS is treatment with azacytidine or decitabine. The major side effect is the early drop in blood counts seen with most chemotherapy drugs. A major benefit is that they need less transfusions and a better quality of life. Even though the drug is not curative it may increases the life span.

With the advent of Lenalidomide (Revlimid; Celgene, Summit, NJ) recently, a new therapeutic option for MDS targeted therapy in cases with isolated 5q deletion was born ([20]). Lenalidomide appears to specifically target the del5q- clone. As mentioned above, the prognosis of MDS with an isolated 5q deletion is generally favourable. Before Lenalidomide however, supportive care was rarely effective, and if effective only for a short period. Lenalidomide has dramatically decreased the necessity of red blood cell transfusions, and is now first-line treatment in IPSS low risk and intermediate risk-1 MDS with del 5q ([20], [21]).

These findings underlie the importance of cytogenetic analysis of MDS BM samples prior to therapy and disease management selection. Future treatment concepts will focus on targeted therapy for other specific MDS entities, as they will by further classified by their respective chromosomal/genomic abnormalities.

1.1.5 MDS pathogenesis

In MDS, the pathogenesis and the molecular basis for its progression to AML remain largely undefined. In general, MDS are considered to be the result of multistep process implicating genetic, epigenetic, and immune-mediated alterations of an (probably) early hemopoietic stem cell (HSC). Furthermore, the current understanding of MDS pathogenesis accepts the acquirement of multiple

consecutive alterations/mutations in an early HSC which may cause a differentiation arrest leading to dysplasia and an increased programmed cell-death (apoptosis) in early (low risk) MDS stages. Whereas subsequent alterations affecting predominantly the myeloid cell proliferation may cause the clonal expansion of aberrant cells combined with decreased apoptosis in advanced (high risk) MDS and progression into AML, finally ([14], [22], [23])(figure 2).

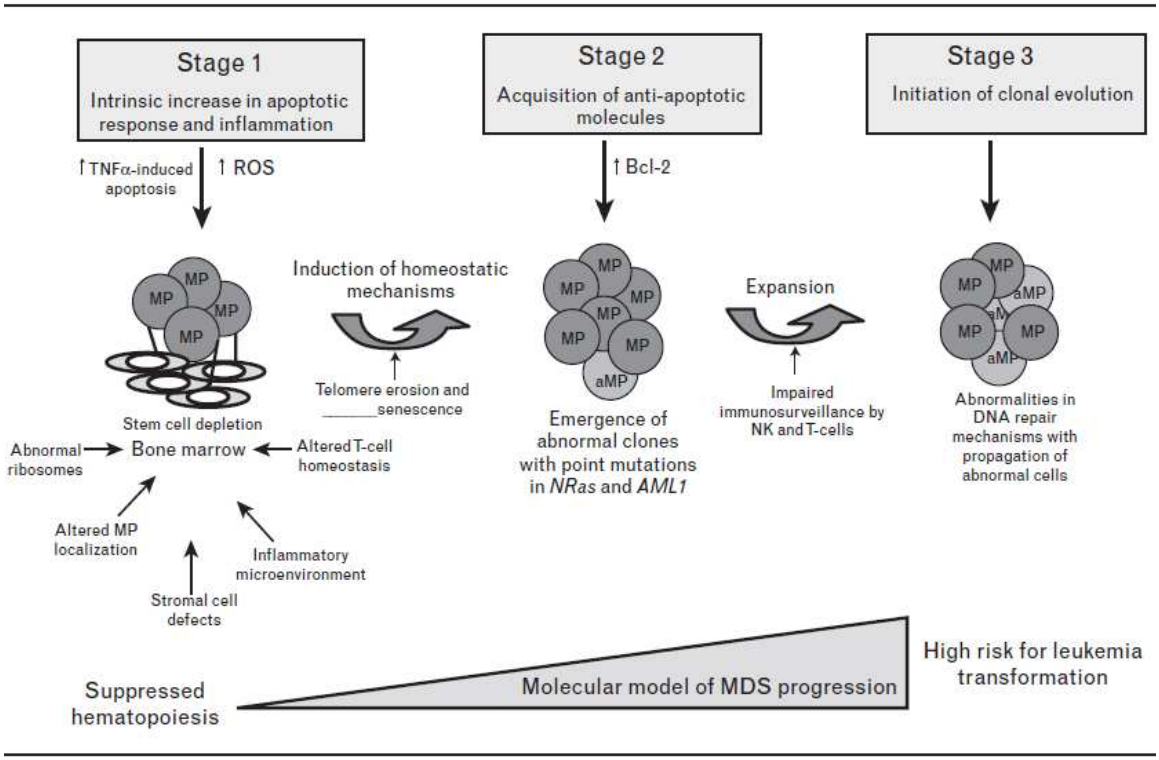


Figure 2. Molecular multistage model of MDS progression: Multiple abnormal events have been linked to intrinsic increases in apoptotic response and the presence of inflammation that converts myeloid progenitors into aMP (abnormal myeloid progenitor) in the bone marrow. These events include intrinsic differences in apoptotic sensitivity within the myeloid progenitors including increased sensitivity to TNFa and abnormal response to ROS. Many distinct environmental insults may modulate the apoptotic response including abnormal ribosomal processing and turnover of progenitors, stromal cell defects, altered location, and homing of myeloid progenitor within the microenvironment and activation of T-cells. In the second stage, data suggest that select myeloid progenitor cells with abnormalities in early-regulatory genes, such as activation and/or mutation of NRas and AML1, lead to acquired survival benefit for some myeloid progenitor clones. The ultimate escape of these abnormal cells may occur through with greater survival and proliferative advantage and/or with the loss of NK immunosurveillance effector mechanisms that then allow the escape of these cells which undergo expansion. The mechanism(s) that control conversion of the pro-apoptotic to antiapoptotic state are completely unknown, but it is plausible that normal pathways of hematopoietic homeostasis may be prompted by the depletion of progenitors during the apoptotic stage leading to increased survival and proliferation associated with replicative senescence and telomere erosion. The third stage, demonstrated by loss in DNA, error-prone DNA repair, loss of genomic stability, and proliferative expansion is highly characteristic of MDS conversion to AML. *Abbreviations:* AML, acute myeloid leukemia; aMP, abnormal myeloid progenitors; MDS, myelodysplastic syndrome; NK, natural killer; ROS, reactive oxygen species. ([24]).

Whether MDS occurs in a cell with only myeloid multipotentiality (i.e., involving megakaryocytic, erythroid and granulocytic/monocytic lineages) or occurs in an earlier progenitor stem cell is not clarified. Emerging data, however, suggest that the BM microenvironment (immune and non-immune components) has an important role in MDS pathogenesis ([24], [25], [26], [27]) and may trigger the clonal evolution and may advance the replacement of normal HSCs to MDS HSCs.

The bone marrow microenvironment is a complex network of cellular components (mainly fibroblasts, adipocytes, osteoblasts, endothelial cells and macrophages), growth factors/cytokines, and extracellular matrix (like fibronectin, collagen type I-IV, laminin, proteoglycans) that *inter alia* supports hematopoietic cell proliferation, differentiation and survival ([28], [29]).

1.2 Mesenchymal stem cells (MSC)

1.2.1 MSC definition

Mesenchymal progenitors (the original term “colony forming unit-fibroblast (CFU-F)” or “marrow stromal fibroblasts (MSF)”), today referred as *mesenchymal stem cells* (MSC), *mesenchymal progenitor cells* (MPC), *adult bone marrow stromal stem cells* (BMSSC) or *marrow stromal cells* (MSC) have been initial described by Friedenstein et al. about 40 years ago ([30]), and are characterized as a cell population of the bone marrow, representing ~1 in 10,000 nucleated cells. MSCs are primitive, undifferentiated, non-hematopoietic stromal cells that gives rise to progenitors for several mesenchymal tissues like bone (osteoblasts), cartilage (chondrocytes), muscle (myocytes), fat (adipocytes), and hematopoietic-supporting stroma (figure 3) ([31]).

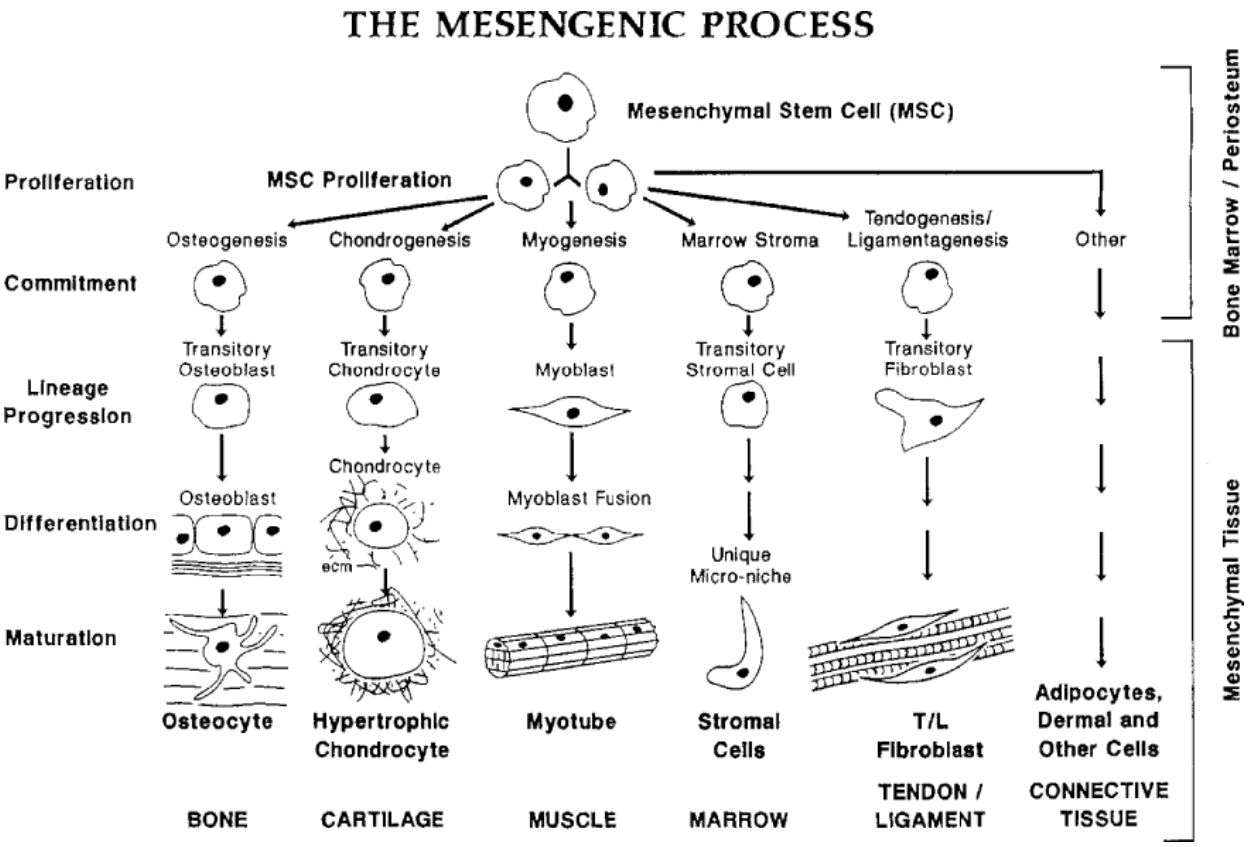


Figure 3. The mesengenic process. Adult mesenchymal stem cells (MSCs) are able to differentiate into bone, cartilage, muscle, marrow stroma, tendon/ligament, fat and other connective tissues in a sequence of lineage transitions. This figure was first drawn to mirror the sequence of events observed in hematopoietic differentiation. The state of knowledge in the late 1980s and early 1990s provided the most information for the lineages on the left and the least information for the pathways on the right ([31]).

1.2.2 MSC characteristics

1.2.2.1 MSC morphology, immunophenotype, and differentiation characteristics

While native BM MSCs are normally non-dividing cells, when isolated and *in vitro* expanded they typically enter a rapidly proliferative state and can be expanded for several passages. The characterization of human MSCs (hMSCs) is based on morphological, functional, and immunophenotypic analysis ([32]). Culture plastic adherence is the hallmark of MSCs typically isolated from the mononuclear layer of the bone marrow (see Material and Methods). The final cultures show a typical morphology of MSCs in a phase contrast microscope, containing narrow spindle-shaped cells.

Immunophenotypically, MSCs express a number of markers but none of these is specific or unique to MSCs. Culture-expanded hMSCs do not express the typical hematopoietic markers CD45, CD34, CD14, or CD11, and are negative for the expression of the costimulatory molecules CD80, CD86, or CD40 or the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule [PECAM]-1), CD18 (leukocyte function-associated antigen-1 [LFA-1]), or CD56 (neuronal cell adhesion molecule-1). But, MSCs have been reported to express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), CD71, and Stro-1 as well as the adhesion molecules CD106 (vascular cell adhesion molecule [VCAM]-1), CD166 (activated leukocyte cell adhesion molecule [ALCAM]), intercellular adhesion molecule (ICAM)-1, and CD29.

Beside their identification by morphology and immunophenotype, MSCs are supposed to differentiate under appropriate culture conditions into bone (osteoblasts), fat (adipocytes), and cartilage (chondrocytes) *in vitro*. As BM MSCs are rare within the fraction of the marrow nucleated cells and while keeping a high renewal capability *in vitro*, it is necessary to work with *in vitro* expanded cells which are relatively easy to maintain.

1.2.2.2 MSC cytokine production

MSCs themselves are able to produce a wide variety of chemokines, cytokines, and growth factors ([33]), and they collaborate with the local environment due to the expression of receptors for above mentioned ones (table 4). Secreted cytokines, together with extracellular matrix production are mediating MSCs hemopoietic support.

Table 4. Main Characteristics of Bone Marrow-Derived Mesenchymal Progenitors: Expression of Specific Antigens, Cytokine Receptors, and Adhesion Molecules, and Production of Cytokines and Matrix Molecules (modified taken by [32]).

Marker type	**Designation**
Specific antigens	SH2, SH3, SH4, STRO-1, α-smooth muscle actin, MAB1740
Cytokines and growth factors	Interleukins: 1α, 6, 7, 8, 11, 12, 14, 15, LIF, SCF, Flt-3 ligand, GM-CSF, G-CSF, M-CSF, SDF1α
Cytokine and growth factor receptors	IL-1R, IL-3R, IL-4R, IL-6R, IL-7R, LIFR, SCFR, G-CSFR, IFNγR, TNFIR, TNFIIR, TGFβIR, TGFβIIR, bFGFR, PDGFR, EGFR
Adhesion molecules	Integrins: αvβ3, αvβ5, Integrin chains: α1, α2, α3, α4, α5, αv, β1, β3, β4 ICAM-1, ICAM-2, VCAM-1, ALCAM-1, LFA-3, L-selectin, endoglin, CD44
Extracellular matrix	Collagen type I, III, IV, V, and VI Fibronectin, laminin, Hyaluronan, proteoglycans

In the context of this study, a short focus on the proinflammatory cytokines: TNFα, IL-1β, and IL 6 as well as on the growth promoting cytokines SDF1 and VEGF is following:

TNFα

Tumor necrosis factor (TNF) α is a pro-inflammatory cytokine mainly synthesized by macrophages but also by monocytes and T cells and found in local tissue microenvironments. TNFα is involved in immune response, systemic inflammation, proliferation, differentiation, tumorigenesis and viral replication ([34]). MSCs express the receptor for TNFα, TNF-R1 (table 4). As a regulatory cytokine it is involved in the pathogenesis of several immune-mediated diseases and hematologic malignancies, including MDS. There it is suggested, that TNFa stimulates the proliferation and the consecutive hypercellularity and inducing apoptosis in advanced MDS stages

(resulting in pancytopenia). Moreover, TNFα seems to involve both hematopoietic and stromal cells ([35]).

IL-1β

Interleukin (IL)-1α and 1β are pro-inflammatory cytokines produced by macrophages, monocytes, and dendritic cells. Both interleukins are involved in immune response, inflammation, and hematopoiesis ([36]). MSCs express IL-1RI, which is the receptor for both IL-1α and IL-1β (table 4). IL-1 probably promotes the growth of blasts in AML (and MDS) due to the unbalanced secretion of IL-1 and its natural receptor antagonist ([37]). Increased levels of IL-1β have been reported in MDS marrow ([38]), and it is suggested that IL-1β is secreted by both, mononuclear and stromal cells ([39]).

IL-6

IL-6 is involved as a pro-inflammatory and an anti-inflammatory cytokine (table 4). It is secreted by T cells and macrophages. IL-6 acts as an anti-inflammatory cytokine due to its inhibitory effect on TNFα and IL-1. It was shown, that IL-6 stimulates and supports the growth of AML blasts ([40]).

SDF1α

Stromal cell derived factor (SDF) 1α is a chemokine produced by stromal fibroblasts involved in immune response, inflammation, hematopoiesis, chemotaxis, and tumor metastasis. SDF-1α provides inflammation and immune response due to the modulation of the lymphocyte chemotaxis (table 4) ([41]). It is suggested, that functional abnormalities of MSC may influence the hemotopoiesis in the bone marrow microenvironment of MDS patients ([42]).

VEGF

Vascular endothelial growth factor (VEGF) is a growth promoting cytokine produced by cells that stimulates the formation of new blood vessels (angiogenesis)(table 4). It is supposed, that VEGF is involved in the MDS pathogenesis due to deregulation of apoptosis and angiogenesis. It has been reported, that autocrine VEGF production in MDS may promote leukemia progenitor self-renewal and inflammatory cytokine elaboration ([43]). Verstovsek *et al.* reported that VEGF expression is the key factor

of the biological behaviour of MDS and AML ([44]). Another recent study disproved this finding by showing no significant increase of VEGF in *de novo* AML compared with controls ([45]).

1.2.2.3 MSC immunomodulatory properties

Beside their hemopoietic supporting and tissue regenerating capacity, human BM MSCs display immunomodulatory properties and reduce inflammation (figure 4). MSCs are so called immunoprivileged as they do not express the major histocompatibility complex (MHC)-II and only intermediate levels of MHC-I while escaping an alloreactive immune recognition ([46]).

The immunosuppressive and immunoregulatory properties of MSCs have been demonstrated both *in vivo* and *in vitro* through modulating the function of immune cells by inhibition the proliferation and function of T cells, B cells, and natural killer (NK) cells ([46]).

Furthermore, it has been observed, that hMSCs suppress lymphocyte alloreactivity *in vitro* in mixed (with mitogens like PHA or IL-2 as they were used in our study) lymphocyte cultures (MLC), independently of the major histocompatibility complex ([47], [48]). But how do MSC suppress T cells is in contrast. One possible mechanisms of T cell suppression mediated by MSC suggests, that activated T cells (due to inflammation, injury or allo-response) produce proinflammatory cytokines such as IFNγ, TNFα, and IL-1α/β which affect MSCs. Briefly, IFNγ in combination with TNFα, IL-1α, or IL-1β seems to activate secretion of chemokines and nitric oxide (NO) produced by MSCs ([49]). It is indicated, that NO from MSCs is involved in the suppression of Stat5 phosphorylation in T cells with a consecutive reduction in T cell proliferation ([50]).

Overall, MSC immunoregulatory functions seem to be mainly mediated through soluble factors rather than cell-to-cell contact interactions. Main MSC-derived immunoregulators are TGF-β1, the hepatocyte growth factor (HGF), IL-10, indoleamine 2,3-dioxygenase (IDO), prostaglandins, and nitric oxide.

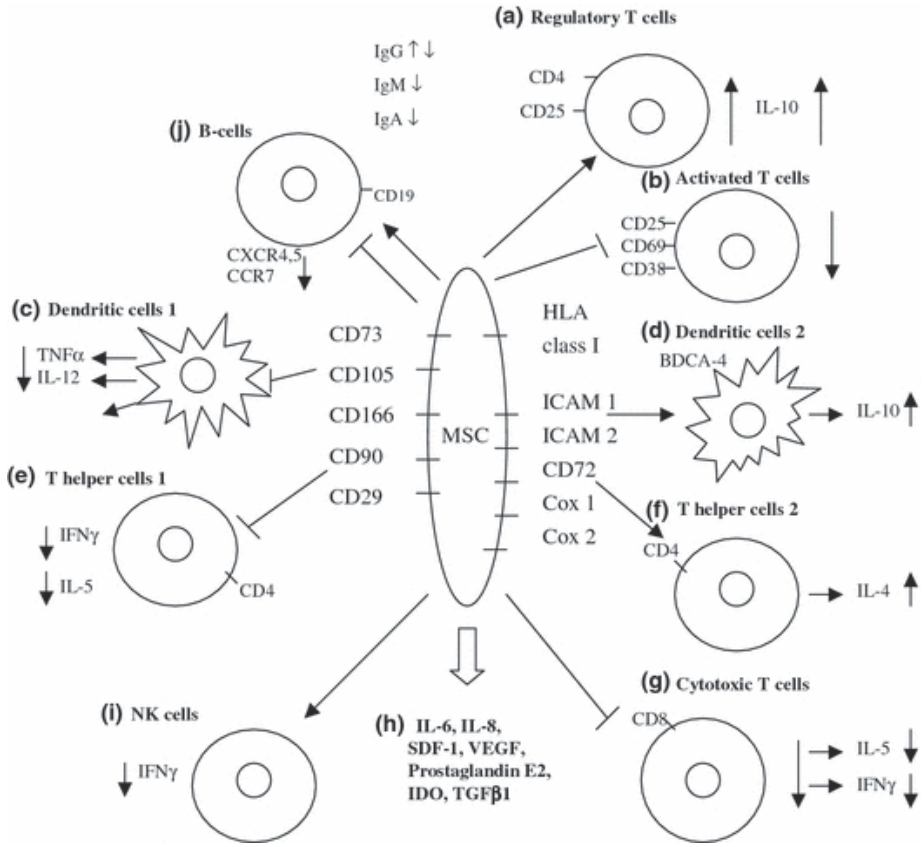


Figure 4. The multiple effects of MSCs on immune cells. (a) MSCs increase the proportion of $CD4^+CD25^+$ cells and IL-10 production. (b) MSCs decrease markers for activated T cells, CD25, CD69 and CD38. MSCs delayed maturation of APC and decreased expression of HLA-DR. (c) Dendritic cell type 1 when stimulated had decreased TNF-α and IL-12, when co-cultured with MSCs. (d) MSCs increased IL-10 secretion by LPS-stimulated dendritic cells type 2, $CD4^+$ cell had decreased IL5-secretion. (e) T-helper cell type 1 IFN-γ production was significantly decreased by MSCs. (f) T-helper cell type 2 increased IL-4 secretion in the presence of MSCs. (g) MSCs inhibit mixed lymphocyte cultures and subsequent development of cytotoxic T cells by a soluble factor. (h) Several soluble factors are produced by MSCs, amongst them are IL-6, IL-8, stem-cell derived factor 1 (SDF1), vascular endothelial growth factor (VEGF). Soluble factors that have been suggested to inhibit T-cell activation are prostaglandin E2, which induces regulatory T-cells, indoleamine 2,3-dioxygenase (IDO), which is induced by IFN-γ which catalyses the conversion from tryptophan to kynurenine and inhibits T-cell responses. Other soluble factors that have been suggested to inhibit T-cell responses are $TGF\beta_1$, hepatocyte growth factor and IL-2. (i) Purified NK cells co-cultured with MSCs significantly decrease IFN-γ levels. (j) MSCs were reported to decrease proliferation and immunoglobulin secretion of B cells when MSC were present in an equal proportion (1:1). Furthermore, chemokine receptor CXCR4, 5 and CCR 7 were significantly downregulated by MSCs. However, MSCs:lymphocytes in a lower concentration (1:10) stimulated B-cell antibody secretion (modified taken from [46]).

1.2.3 MSC applications

Bone marrow derived mesenchymal stem cells (BM MSCs) show another promising therapeutic potential in hematologic diseases. The clinical application of MSCs is in progress and is nowadays mainly used for prevention and treatment of therapy-resistant acute graft-versus-host disease (GvHD), tissue repair (Osteogenesis

imperfecta ([51], [52]), myocardial infarction ([53])) and treatment of autoimmune diseases ([54]). Clinical studies have demonstrated the safety and efficacy of BM MSCs in graft versus host disease (GvHD) ([31] [55]).

The observation that graft versus leukaemia (GVL) activity but not the conditioning protocol toxicity is the probably most important factor for disease eradication in at least some allogeneic HSCT indications has led to the development of modified conditioning regimes with reduced intensity. It is possible, to improve the GVL effect by giving donor T-lymphocyte infusions ([46], [56]). Thus, previously limitations due to high risks for transplant related mortality such as patient age and comorbidity have been modified. However, the still major transplant related complication is GVHD. In some cases with failed HSCT, the transplantation of allogeneic T-lymphocytes is responsible. It is particularly shown, that immunosuppressive drugs as well as MSC can suppress the clonal expansion and activity of cytotoxic T-lymphocytes and may decrease the mortality through GvHD ([47], [55], [56]). Autologous BM MSC transplantation represents therefore an investigational therapeutic strategy in patients with MDS undergoing an allogeneic stem cell transplant procedure.

1.3 MSCs in MDS

As stromal growth and hemopoiesis in MDS is seriously impaired, the dysregulated interactions can be the result of alterations in the bone marrow matrix composition itself (including relative deficiency of hematopoietic growth factors and/or aberrant release of inhibitors) or of abnormal function and/or expression of cell surface adhesion receptors on the progenitor cells (HSCs), or both. And, as excessive apoptosis as a result of ineffective hematopoiesis in MDS is not restricted to MDS clone but affects also the normal hematopoietic cells, once more a possible apoptosis-inducible effect of the marrow microenvironment is suggested. And indeed, TNFα, IL-1β, IFNγ, TRAIL, and TGFβ are pro-apoptotic cytokines all up-regulated in MDS BM ([57]; [58]; [38]; [59]). Otherwise, the growth-promoting cytokines, such as erythropoietin (EPO), IL-3, IL-6 and thrombopoietin (TPO), are identified to be increased in MDS BM ([39, 60], [61]).

As a short summary of available data of MSCs in MDS is following, two facts have to be stressed out. A) In most of the studies with MSCs in MDS it was necessary to work with *in vitro* expanded cells. As there is no unique cellular marker for MSC, a possible explanation for the confusion in research data is at least attributed to different methodology for MSC isolation used. B) Another important fact is that in most of the in general rare studies existing and described below, MDS patients were studied as an entity rather than MDS subtypes according to the WHO classification, which can easily lead to data misleading explanations.

Cumulative data are in agreement with a normal frequency in the marrow, and with a rather normal *in vitro* phenotype ([61]; [62]; [63]; [64], [65]) and normal differentiation capacity ([66]; [63], [65], [26]). Most of studies agree on a defective MSC *in vitro* proliferative potential, possibly due to previous exposure of cells to an inflammatory environment within patients' marrow ([61], [26], [65], [66]). The investigation of cytokine levels in MSCs cultures show that the main intrinsic MSC cytokine production (TNFα, IL-1β, IL-6, SCF, GM-CSF, SDF-1, and VEGF) and extracellular matrix deposition (fibronectin and collagen) are within the normal limits in patient cells ([63]). However upon TNFα and IL-1β treatment, a GM-CSF induction was described *in vitro* ([63]), suggesting a potential deregulation in MSC cytokine production under inflammatory conditions of MDS marrow. So far, for MDS MSCs a reduced *in vitro* immunosuppressive capacity against mitogen- or allo-activated T-cells is reported ([62], [64]). Lastly, the cytogenetic profile (table 5): Some research groups report that patient MSCs display normal cytogenetics ([66], [27]; [62]), while others show the presence of clonal (and non-clonal) chromosomal aberrations ([61], [67]; [63], [65]). Recently for the first time, fresh non-expanded patient BM MSCs were analyzed using array-based comparative genomic hybridization (array-CGH) and fluorescence *in* situ hybridization (FISH), harboring genomic changes, once more distinct from the ones found in hematopoietic cells (HCs) ([65]).

Table 5. Cytogenetic analyses of BM MSCs in MDS patients

Study	Patients (n=)	CCA*	FISH	Array-CGH*	Type of aberrations found
[66]	4	n.d.	+	n.d.	-
[61]	9	5/9 (56%)	n.d.	n.d.	Numerical (losses)
[27]	9	n.d.	+	n.d.	-
[62]	15	0/15 (0%)	n.d.	n.d.	-
[67]	16	7/16 (44%)	+	n.d.	Structural (balanced/unbalanced) and numerical (gains/losses)
[63]	12	8/12 (67%)	n.d.	n.d.	Not described
[65]	17	n.d.	+	17/17 (100%)	Structural (unbalanced gains more than losses)

*In CCA and array-CGH the ratio corresponds to the number of patients with genetic aberrations within the total number of patients, and the percentage is given in parenthesis. Abbreviations: CCA: classic cytogenetic analysis; FISH: fluorescence *in situ* hybridization; array-CGH: array-based comparative genomic hybridization; n.d.: not done

1.4 Aim of the study

Hemopoiesis is viewed as a hierarchical system with the hematopoetic stem cell at the origin (figure 5)([68]).

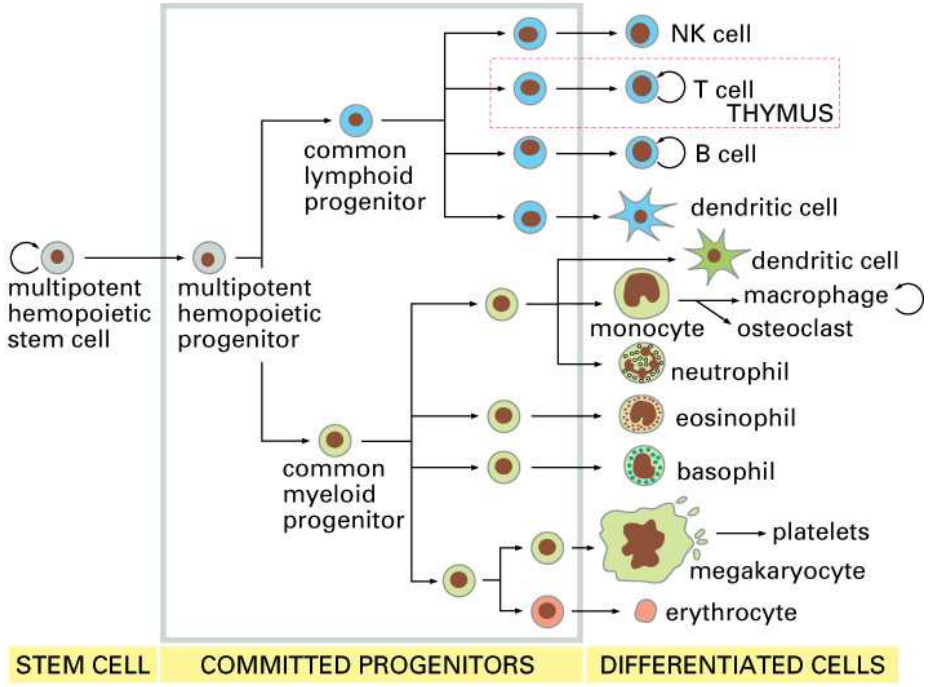


Figure 5. Cell hierarchy (Hemopoiesis schematic representation). Highly simplified diagram to demonstrate the stages of development of various cell types (taken from: Molecular Biology of the Cell, Fourth Edition: Bruce Alberts/ Alexander Johnson/ Julian Lewis/ Martin Raff/ Keith Roberts/ Peter Walter).

The hematopoiesis supportive cellular components of BM stroma (adipocytes, osteoblasts, and fibroblast-like cells) derive from a common progenitor cell, the mesenchymal stem cell (MSC) ([69], [70], [71]). MSCs do not show such a hierarchical structuring so far [68]. However, since a common stem cell with wide differentiation potential, namely the multipotent adult progenitor cell (MAPC), has been proposed for BM HSCs and MSCs (figure 6) ([72], [73]), it seems reasonable to assume that BM MSCs in MDS patients might belong to the abnormal clone and might be primarily defective.

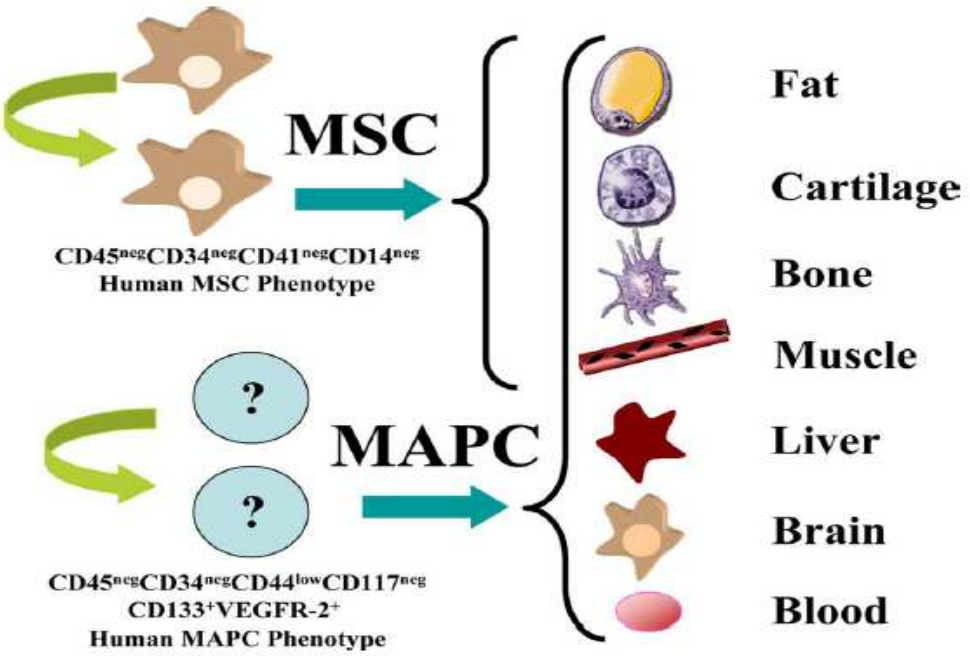


Figure 6. Two types of mesenchymal stem cells are found in adult bone marrow. The mesenchymal stem cell (MSC) is mesodermally restricted and forms primarily connective tissue types (cartilage, fat, bone, tendon, muscle) and can self-renew to maintain the stromal compartment. The multipotent adult progenitor cell (MAPC) appears to have the greatest differentiation potential of all stem cells found within the bone marrow cavity and has substantial overlap in differentiation potential with MSCs. MAPCs have been reported to have the capacity to contribute to progeny of all three embryonic germ layers: liver derived from endoderm, brain derived from ectoderm, and muscle and blood derived from mesoderm. The MAPC thus can give rise to tissues that comprise that of the MSC and additional tissues with the notable inclusion of blood (documented for mouse MAPCs). The phenotypes shown are for human MSCs and MAPCs, although mouse and rat cells having these properties have also been characterized. The question marks indicate the unknown provenance of MAPCs ([73])

Alternatively, BM MSCs in MDS patients might display secondary genetic aberrations and/or functional defects due to the deranged release of proinflammatory mediators and inhibitory cytokines by the apoptotic hematopoietic cells and the activated cellular components of the BM microenvironment.

The possible involvement of the BM stroma progenitors (namely MSCs) in the pathogenetic/pathophysiologic process of MDS has not been extensively studied whereas existing data on the cytogenetic and functional integrity of BM MSCs in MDS patients have shown contradictory results.

In this context we have studied the reserves and qualitative characteristics of BM MSCs in MDS patients (n=13) in comparison to healthy individuals (n=20) in terms of the proliferative and clonogenic potential, the differentiation capacity and immunoregulatory properties in order to explore whether the MSCs are primarily affected in MDS. The cytogenetic characteristics of BM MSCs in comparison to BM hematopoietic cells as well as the chromosomal stability of BM MSCs during

passages have been investigated. The chromosomal stability of BM MSCs in different passages *in vitro* is of interest, since there are clinical purposes.

The results of the study might be helpful and important for the possible use of MSCs in MDS patients for therapeutic purposes, and for the better understanding of the pathophysiology of the disease, since for example common chromosomal abnormalities might imply a defect in a common stem cell.

2. Material and Methods

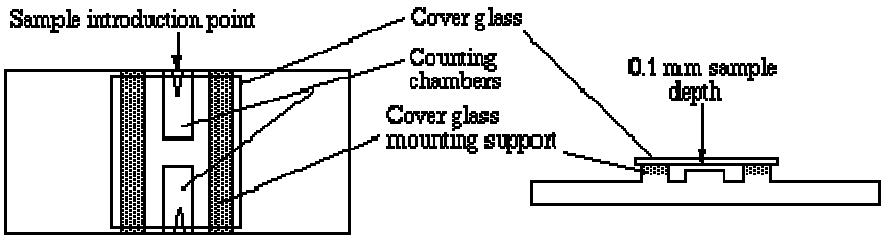
2.1 Patients and controls

In the present study, bone marrow nuclear cells obtained from the iliac crest of thirteen patients with *de novo* MDS, 7 female and 6 male patients with a median age of 72 years (range 62 to 84 years), and 20 age- and sex-matched healthy individuals were studied. Patients were classified according to the World Health Organization (WHO) and the International Prognostic Scoring System (IPSS) and were studied on diagnosis before receiving any medication. According to the WHO subtype classification system, 6 patients were categorized as RA, 3 patients as RAEB-1, and 4 patients as RAEB-2, respectively. On the basis of the IPSS score risk category, 4 patients each were classified as low risk, Intermediate-1 risk, and Intermediate-2 risk, and 1 patient with high risk. The study has approved by the Ethics Committee of the University Hospital of Heraklion and informed consent according to the Helsinki Protocol was obtained from all subjects. Patient clinical and laboratory characteristics are summarized in table 7. 5 male donors with a median age of 74 years (range 62 to 83 years) were analyzed by classical cytogenetic analysis (CCA) and their characteristics are summarized in table 9.

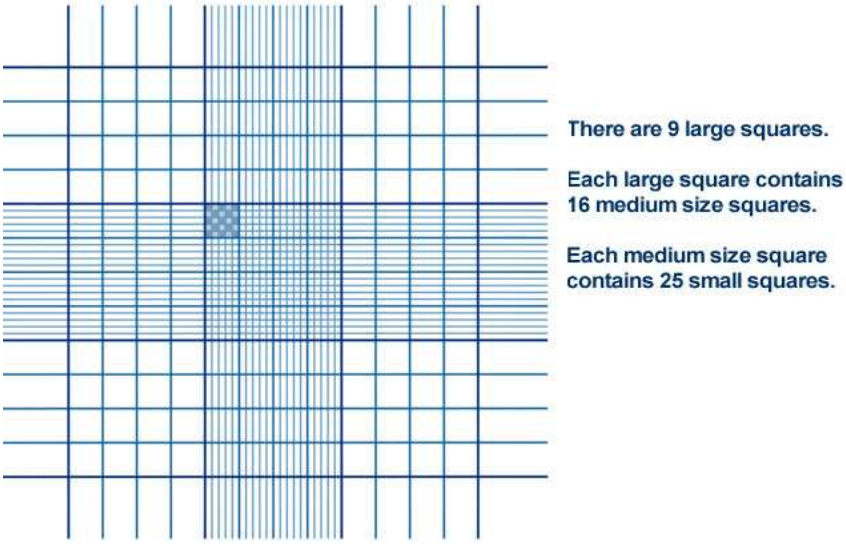
2.2 Mesenchymal stem cell cultures

Bone marrow cells obtained from the posterior iliac crest aspirates were immediately diluted 1:1 in Dulbecco's Modified Eagle Medium-Low Glucose (DMEM-LG; Gibco Invitrogen, Paisley, Scotland), supplemented with Heparin (Sigma, Saint Louis, MO), 10% fetal calf serum (FCS; Hyclone, Logan, UT), and 100 IU/ml Penicillin/Streptomycin (PS; Gibco). Diluted BM sample were centrifuged on Lymphoprep (Nycomed Pharma, Oslo Norway, density 1077g/cm^3) at 1800rpm for 30min without brake at room temperature (RT). The isolation of BMMCs requires separation of bone marrow cells by centrifugation through a density gradient. Whole bone marrow is layered onto an aqueous medium containing ficoll and sodium diatrizoate at a predetermined density of 1.077 g/ml at 25°C. Gentle centrifugation at room temperature results in the separation of BMMCs at the serum medium ficoll

interface (including MSC) with neutrophils, platelets, and red blood cells (RBCs) passing through the interface and collecting at the bottom of the tube. The BMMC interface is then collected and 2x washed with sterile DMEM-LG (1mg/ml PS, 10% FCS) to remove any contaminating separation medium. Finally, 15µl of the BMMCs pellet was diluted with 15µl Trypan Blue. Then 15µl of this mix were put down of the cover slide of the Neubauer chamber slide:



The BMMCs were counted at a light optical microscope (40x objective) in the 16 medium size squared area as shown below:



Each square has a surface area of one square mm, and the depth of the chamber is 0.1 mm. Thus, the BMMCs concentration is the product of the counted cell number$x2x10^4$/ml.

Then, $5x10^6$ BMMCs supplemented with 5ml DMEM-LG (10% FCS, 1mg/ml PS) were cultured in $25cm^2$ culture flasks at 37^oC-5%CO_2-100%humidity, so called passage 0 (P0). The MSC adhere to the culture flask while essentially all other cells

are nonadherent and were removed by rinsing ([30]). MSC grew and expanded in culture, yielding a well-defined population of pluripotent stem cells. The next day, after a wash procedure with 10ml PBS (Phosphate buffered saline, 4^{o}C, pH 7.2-7.4) and nonadherent cells were rejected, DMEM-LG was replaced by fresh ones (5ml). Then, the cells were growing until a 70-90% confluence (approximately 10-20days) including a re-feed of the flasks every three days with a full change of DMEM-LG complete medium. For cell harvesting and ongoing to passage 1 (P1), the old culture medium was removed from the flask, 2-3ml PBS supplemented with 1ml 0.25% trypsin-1mM EDTA (Gibco) was added and incubated for 3-7 min at 37ºC in a 5% CO_2 incubator with humidified air. Afterwards, the flasks were checked for cell detachment. When cells were detached, an equal volume of DMEM-LG plain was added. After centrifugation at 1680rpm for 7min 5x10^{5} cells/ml were cultured in 75cm^{2} with 10ml DMEM-LG complete until a confluence of 70-90%. On 70%–90% confluence, cells were detached using 0.25% trypsin–1 mM EDTA and re-seeded for a total of 8 passages (P).

Cell-free supernatants were stored at −70°C for measurement of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, stromal-derived factor (SDF)-1α, and vascular endothelial growth factor (VEGF) levels by an enzyme-linked immunosorbent assay (ELISA). All ELISA kits were purchased from R&D Systems (Quantikine, Minneapolis, MN) except the TNF-α kit (Biosource International Inc., Camarillo, CA).

For chromosomal analysis, at passage 2, 300µl Colcemide over night was added. The next day, cells were detached using 0.25% trypsin–1 mM EDTA and the procedure was following the protocol in *2.3 Chromosome preparation and cytogenetic analysis of BM hematopoietic cells* at step: Chromosome harvesting and fixation in all 13 MDS patients and in 5 healthy individuals. In 6 MDS patients, a chromosome analysis was also performed in MSCs from P4, P6, and P8. Whenever possible, 15–20 metaphase cells in each passage (range 0–40, median 13) were analyzed and classified according to the ISCN 2005 (An International System for Human Cytogenetic Nomenclature: eds. Shaffer LG & Tommerup N, S. Karger, Basel, Switzerland).

2.2.1 Differentiation assays

To fulfil the International Society for Cellular Therapy (*www.**celltherapysociety**.org*) requirements for MSC definition, the multipotential of MSCs was checked by their ability for differentiation into adipocytes, osteoblasts and chondrocytes.

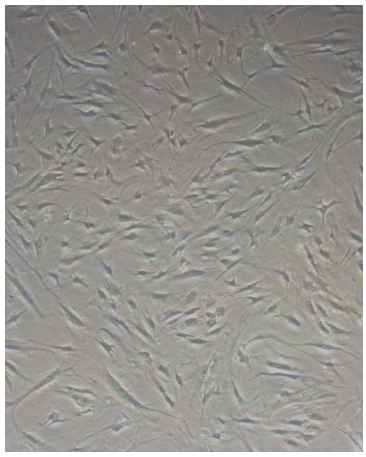


Figure 7. Typical spindle like morphology of MSCs at passage 2 *in vitro* in a phase contrast microscope.

2.2.1.1 Induction of adipogenic differentiation

Adipogenic differentiation was induced following a 21-day culture (37^{o}C/5% CO_2 fully humidified atmosphere) of cells from passage 2 (figure 7) at a density of 80x10^3 cells/cm^2 in 60-mm plates in MSC medium supplemented with 10% FCS / 0.5mM 1-methyl-3-butylisoxanthine / 1 μM dexamethasone (Dex) / 0.2 μM indomethacin / 10 μg/mL insulin. Adipogenesis was assessed by Oil Red O staining measuring neutral lipid accumulation in fat vacuoles. In detail, removed cells were washed 2x with PBS and fixed in 2% paraformaldehyde (PFA)/ 0.075M L-Lysine dihydrochloride/ 0.0375M NaH_2PO_4 for 15 minutes. Fixative was removed and cells were washed again 2x with PBS. Staining was performed for 20 minutes in 60% Oil Red O stock solution (0.5% in 99% isopropanol) diluted in distilled H_2O (dH_2O). After 2x washes with dH_2O, cells were ready for morphological evaluation. MSC adipogenic differentiation potential was also assessed by RT-PCR for the determination of adipocyte-associated gene expression: adipose fatty acid-binding protein (aP2) and peroxisome proliferator activated receptor-γ (PPAR- γ). In detail, total RNA isolated according to the manufacturer's protocol from differentiated MSCs from patients and controls

(RNeasy mini kit; QIAGEN, GmbH, Hilden, Germany) was reverse-transcribed (SUPERSCRIPT II; Gibco) and amplified by polymerase chain reaction (RT-PCR) for the evaluation of specific, differentiation-associated gene expression, i.e. here adipocyte-associated gene expression of aP2 and PPAR- γ.

RT-PCR specific conditions and primer sequences

Gene	Primers	Annealing temperature	Cycles
aP2	Forward: 5′-GTACCTGGAAACTTGTCTCC-3′ Reverse: 5′-GTTCAATGCGAACTTCAGTCC-3′	60 °C	29
PPARγ	Forward: 5′-GAATGTCGTGTCTGTGGAGA-3′ Reverse: 5′-TGAGGAGAGTTACTTGGTCG-3′	60 °C	29
β2m	Forward: 5′-TCCAACATCAACATCTTGGT-3′ Reverse: 5′-TCCCCCAAATTCTAAGCAGA-3′	60 °C	26

Products were normalized according to β2 -microglobulin (β2m) expression, using the ImageJ densitometry analysis system.

2.2.1.2 Induction of osteogenic differentiation

Osteogenic differentiation was induced following a 21-day culture (37°C/5% CO_2 fully humidified atmosphere) of cells from passage 2 (density of $15x10^3$ cells/cm^2 in 60-mm plates) in MSC medium supplemented with 0.1 μM Dex / 0.15 mM ascorbate-2-phosphate / 3 mM NaH_2PO_4. Osteogenesis was assessed by alkaline phosphatase (ALP) activity and mineralization was checked by using Von Kossa staining. In detail, removed cells were 1x washed with PBS (ice cold) and fixed in 10% neutral buffered formalin (NBF) for 15 minutes. Afterwards, cells were 2x washed in dH_2O for 1 min and other 15 min, and stained for 45 min in 0.1mg/ml naphthol AS MX-PO_4 dissolved in N,N dimethylformamide, in 1M Tris-HCl pH 8.3, containing 0.5mg/ml Red Violet LB salt. Sites of mineralization were identified using 0.25% w/v silver nitrate in dH_2O for 30 minutes (Von Kossa staining). After a final wash with dH_2O for 3 times, cells were

ready for morphological evaluation. MSC osteogenic potential was again assessed by RT-PCR for the evaluation of osteoblast-associated gene expression, i.e. ALP and runt-related transcription factor 2 (RUNX2).

RT-PCR specific conditions and primer sequences

Gene	Primers	Annealing temperature	Cycles
ALP	Forward: 5′-CTGGACCTCGTTGACACCTG-3′ Reverse: 5′-GACATTCTCTCGTTCACCGC-3′	60 °C	29
RUNX2	Forward: 5′-TTACTTACACCCCGCCAGTC-3’ Reverse: 5′-TATGGAGTGCTGCTGGTCTG-3	60 °C	29

2.2.1.3 Induction of chondrogenic differentiation

For chondrogenic induction, MSCs from passage 2 were pelleted in 15ml polypropylene tubes ($25x10^4$ cells/pellet) and cultured for 21 days (37^oC/5% CO_2 humidified chamber) in DMEM-High Glucose (Gibco) without Serum, supplemented with 6.25 μg/mL insulin / 6.25 μg/ml transferring / 1.33 μg/ml linoleic acid / 1.25 mg/ml bovine serum albumin / 1 mM sodium pyruvate / 0.17 mM ascorbate-2-phosphate / 0.1 μM Dex/0.35 mM l –proline / 6.25 ng/ml selenous acid / 0.01 μg/ml transforming growth factor-β 3 (R&D Systems). Chondrogenesis was assessed by Alcian blue and Masson’s trichrome stains. In detail, removed cells were paraffin sectioned, fixed in 70% ethanol and stained for 10 minutes with 1.5% w/v alcian blue, 1.5% v/v acetic acid in dH_2O. Following de-staining, sections were evaluated morphologically. MSC chondrogenic potential was also assessed by RT-PCR for the determination of chondrocyte-associated gene expression, i.e. collagen type II (COL2A1) and aggrecan (AGC1). (For chondrocyte RNA isolation, a Proteinase K (QIAGEN) initial step was introduced according to the manufacturer’s protocol.)

RT-PCR specific conditions and primer sequences

Gene	Primers	Annealing temperature	Cycles
COL2A1	Forward: 5′-AACTGGCAAGCAAGGAGACA-3′ Reverse: 5′-AGTTTCAGGTCTCTGCAGGT-3′	57 °C	29
AGC1	Forward: 5′-ATGCCCAAGACTACCAGTGG-3′ Reverse: 5′-TCCTGGAAGCTCTTCTCAGT-3′	57 °C	29

2.2.2 Immunophenotypic characteristics of MSCs

Beside their characteristic morphology, MSCs have to be and were characterized by flow cytometry as well. As already mentioned, MSCs express the following markers: CD29 (130kDa integrin b1 chain), CD44 (H-CAM), CD73 (ecto-5'-nucleotidase), CDw90 (Thy-1), CD105 (Endoglin), and CD146 (Mel-CAM). And, MSCs are negative for typical hematopoietic markers: CD45, CD14, and CD34. In brief, trypsinized MSCs from P1–P6 were immunophenotypically characterized following a standard protocol given by the manufactur and using monoclonal antibodies against CD29 (4B4; Cyto-Stat/Beckman-Coulter, Fullerton, CA), CD44 (J173; Immunotech/Coulter, Marseille, France), CD73 (AD2; Becton Dickinson-Pharmingen, San Diego, CA), CD90 (F15.42; Immunotech/Coulter), CD105 (SN6; Caltag, Burlingame, CA), CD146 (P1H12; Becton Dickinson-Pharmingen), CD45 (IMMU19.2; Immunotech/Coulter), CD14 (RMO52; Immunotech/Coulter), and CD34 (QBend10; Beckman-Coulter). Data were processed in an Epics Elite flow cytometer (Coulter, Miami, FL).

2.2.3 Clonogenic potential of MSCs

Colony-forming unit fibroblast assay.

A colony-forming unit fibroblast (CFU-F) assay was used for the evaluation of the clonogenic potential of MSCs through P1–P6 ([74]). Trypsinized MSCs from P1 to P6 were seeded at 3 different concentrations (50–150 cells) in 60-mm plates for 14

days. CFU-Fs were quantified using Giemsa staining (Gronthos S, SE Graves and PJ Simmons.(1998): Isolation, purification and in vitro manipulation of human bone marrow stromal precursor cells. In: Marrow Stromal Cell Culture. Beresford JN, ME Owen, eds. Cambridge University Press, Cambridge, pp 26 – 42). The number of CFU-F was estimated per 100 MSCs on the basis of the linear regression analysis obtained from the 3 different initial cell concentrations ([74]).

Limiting dilution assay.

A limiting dilution assay (LDA) was used to indirectly evaluate the frequency of MSCs within BMMCs following 6-week culture of 7 different concentrations of BMMCs (250–10.000 cells/well) in 96-well flat bottom plates following a standard protocol in our laboratory ([75], [76]). Wells with >50 adherent spindle-shaped cells were considered positive and MSC frequency corresponded to the dilution resulting in 37% negative wells ([77]).

2.2.4 Proliferative potential of MSCs

The proliferative potential of MSCs was evaluated by a methyl triazolyl tetrazolium (MTT)-based assay at P2 and also by estimating the population doubling time through P1–P6 [32]. The formula 2 n = *N* x / *N* 0 was used for the calculation of the population doublings (*n*) at each passage based on the number of cells counted in the flask after trypsinization (*N* x) and the number of cells initially plated (*N* 0).

2.2.5 T-cell proliferation assay

PB samples from healthy donors were centrifuged on Histopaque-1077 to obtain the mononuclear cells and the $CD3^+$ cell fraction was fractionated by indirect magnetic labeling according to the manufacturer's protocol (magnetic-activated cell sorting; MACS isolation kit, Miltenyi Biotec GmbH, Germany). In each experiment, purity of $CD3^+$ cells was >96% as estimated by flow cytometry. To evaluate the capacity of MSCs from MDS patients and healthy subjects to suppress T-cell proliferative responses, we stimulated 5×10^4 immunomagnetically sorted normal $CD3^+$ cells with

phytohemagglutinin (PHA; 2 µg/mL) or interleukin-2 (IL-2; 500 IU/mL) in the presence or absence of 10^4 irradiated (30 Gy) BM MSCs from MDS patients or allogeneic healthy controls in V-bottomed 96-well culture plates for 7 days in 0.2 mL RPMI-1640 medium (Gibco) containing 10% FCS ([78], [79]). T-cell proliferation was measured on day 7 following an 18-h pulse with 1 µCi/well ^{3}H-thymidine (^{3}H-TdR; Amersham, Buckingham, UK). ^{3}H-TdR incorporation was measured by using a liquid scintillation counter (LS1701 beta counter-Beckman, USA). The percentage of inhibition of T-cell proliferation by MSCs was calculated by dividing the difference of counts per minute (cpm) between cultures of T cells with and without MSCs in the presence of the activator by the cpm obtained from the cultures of T cells with the activator alone. Experiments were performed in triplicates.

2.3 Chromosome preparation and cytogenetic analysis of BM hematopoietic cells (and culture expanded MSCs)

Whole bone marrow (500 and 1000µl each) supplemented with Heparin from MDS patients and healthy controls were cultured in culture tubes with 5ml RPMI-1640 medium supplemented with 5% FBS and 100 IU/ml Penicillin/Streptomycin at 37°C for 24 and 48 hours. For cell cycle arrest, the next day Colcemide (0.1 g/ml) was added for 2 and 24 hours before harvesting:

culture tube	day 0	day1	day2
1:	500µl BM	colcemide (2h) +fixation	
2:	1000µl BM	colcemide (2h) +fixation	
3:	500µl BM	colcemide (24h)	fixation
4:	1000µl BM	colcemide (24h)	fixation

Chromosome harvesting and fixation: First, a culture tube centrifugation at 1000rpm for 7min, supernatant-warping and resuspension was done. Under culture tube shaking conditions 10ml of a hypotonic KCl solution (5.592g KCl/1l Aqua dest.) was added (drop by drop) for 20min (Chromatin swelling and hydrolysis of the red blood cells). Afterwards, culture tube centrifugation at 800rpm for 10min, supernatant-warping, resuspension and under culture tube shaking conditions a fixation with a mixture of 10ml glacial acid and absolute methanol (1:3, Carnoy's Fixativ) (drop by drop) for 30min was done. Following a culture tube centrifugation at 1000rpm for 7min at 4°C, the fixation with glacial acid and absolute methanol was repeated another two times. After a last supernatant-warping and resuspension, the cell suspensions were stored at -20 °C. For chromosome analysis, about 6-8 drops of each culture suspension were droped on cold (4°C) and wet slides and air-dry for at least 24h (aging). For banding, a modified chromosome-banding technique (GAG; Giemsa bands by acetic saline-Giemsa) was used including slide incubation in 2xSSC for 12-16h at 60°C and Giemsa staining for 3 min at room temperature followed by H_20 washing for 30s and air dry.

In each case, 15 to 25 metaphase cells (range 5 to 25) taken by a high resolution CCD camera on a Axioskope2plus microscope (Zeiss) were analyzed. The karyotype was generated on screen with the Ikaros Karyotyping System (Metasystems) (figure 8) and evaluated according to the ISCN.

A chromosomal aberration was defined as clonal abnormality when at least 2 metaphases were demonstrating the same structural rearrangement or chromosome gain, whereas a chromosome loss had to be identified in at least 3 metaphases (see below section *2.6 Terminology*).

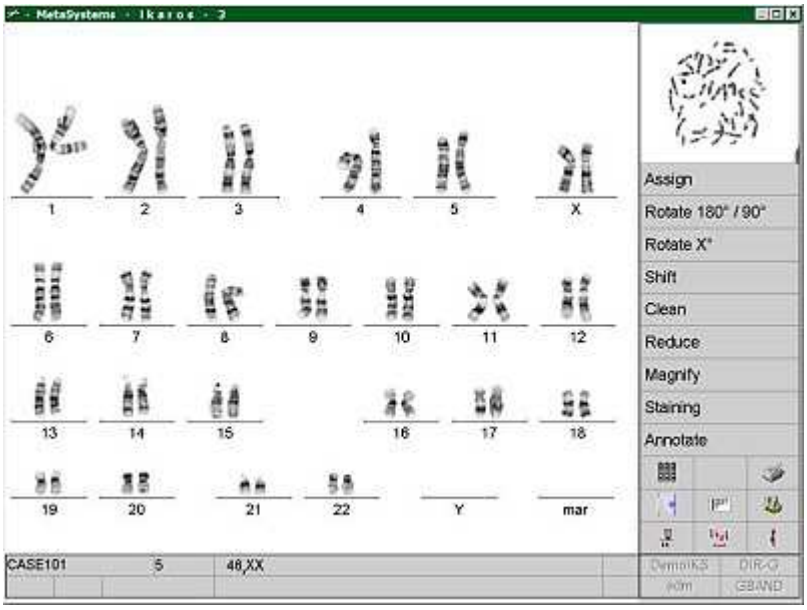
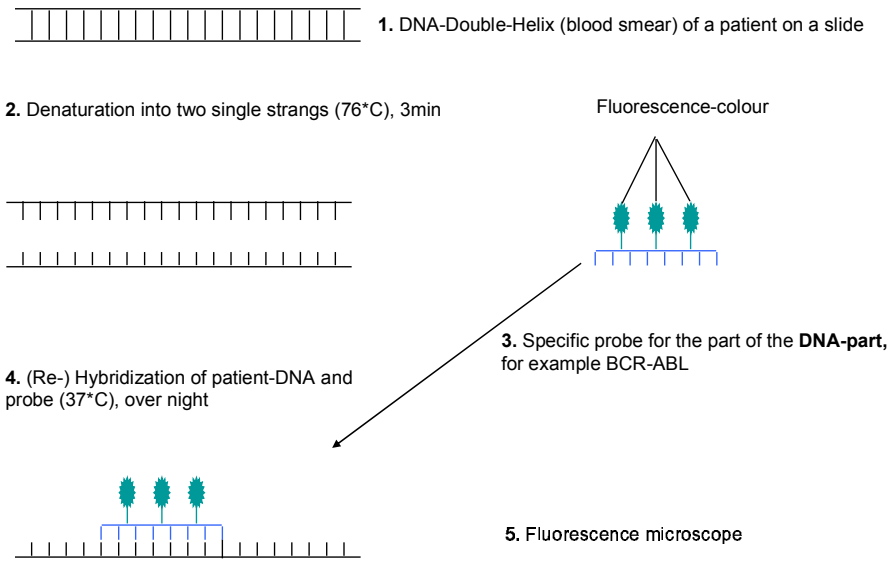


Figure 8. On screen chromosome analysis with the Ikarus Karyotyping System showing an example for one metaphase chromosome set in G banding: 46,XX

2.4 Fluorescence in situ hybridization (FISH)

In general, fluorescence *in situ* hybridization (FISH) is a powerful molecular cytogenetic technique that gives the possibility to detect single or all chromosome pairs, specific regions/parts or even single genes on chromosomes and allows the detection (or confirmation) of numerical (whole chromosome gains and/or losses) or structural chromosome aberrations such as translocations, deletions or amplifications ([80]). FISH uses fluorescent molecules to vividly paint genes or chromosomes. In addition to CCA, FISH (with locus-specific probes, LSI probes, see below), is not restricted to metaphase cells (as it can be performed in interphase cells as well) and allows the detection of submicroscopic aberrations ([81]).

The principle is based on the ability of single-stranded DNA (commercial available or preparable short sequences of single-stranded DNA, so called probes, which are complementary to the DNA sequences the researchers wish to paint and examine) to anneal to complementary DNA (patient/test-DNA):



Briefly, metaphase or interphase cells of bone marrow or peripheral blood are fixed on slides **(1)** and are denaturated **(2)**, whereby the double-strand DNA (test-DNA) is separated into two single strand DNAs. Adding now 1.5µl of the fluorochrome-labeled target-DNA (nowadays commercial available probes) and putting a cover slide and closing with Fixogum (Marabu, Tamm, Germany) **(3)**, a new double strand DNA consisting of test- and target-DNA by (Re-) Hybridization over night is formed **(4)**. The next day, the cover slide was removed and the slide was washed in 0.4x SSC for 2min at 72^{o}C and immediately in 2x SSC/0.1%TritonX100 for 1min at RT, and immediately 10µl DAPI-Antifade (Abbott Laboratories, Illinois, USA) were added. DAPI (4',6-Diamidin-2'-phenylindoldihydrochlorid) is used to visualize the DNA in the nucleus (blue), and antifade (1,4-phenylene-diamine) is used to prevent the signal loss. In our cases we used directly labeled probes (spectrum green and/or spectrum orange), which can be directly identified with a fluorescence microscope **(5)**, and the number and location of the hybridization signals represent the target sequences in the chromosomal DNA that are complementary to the probe.

Depending on the type and size of the probes, three different types of FISH probes with different application are available:

a) Locus-specific (LSI) probes: hybridize to a particular region of a chromosome (for example, BCR-ABL)

b) Centromeric repeat (cep) probes: generated from repetitive sequences found at the centromeres of chromosomes (for example an extra copy of a chromosome 21, trisomy 21)

c) Whole chromosome probes: a whole chromosome pair is visualzed, useful for examining chromosomal abnormalities, for example, when a part of one chromosome is attached to the end of another chromosome or for the identification of so called marker chromosomes

While LSI and cep probes can be used in interphase and metaphase chromosomes, the latter one can just be performed on metaphases.

2.4.1 LSI probes

FISH in the present study was performed using commercially available locus-specific probes for chromosome 5 [LSI EGR1(5q31)/D5S23,D5S721], chromosome 7 [LSI D7S486(7q31)/CEP7], and chromosome 8 (CEP8, SpectrumOrange) following the protocol of the manufacturer (Abbott Laboratories, Illinois, USA). The signals of at least 100 interphase nuclei in each case were visualized and counted with a Zeiss Axioskop microscope (Zeiss, Jena, Germany) with filter for DAPI (blue), FITC (SpectrumGreen), and TexasRed (SpectrumOrange), and documented with the analyzing system ISIS (MetaSystems, Altlussheim, Germany) and documented by CCD (Charge Coupled Device)-camera (ISIS-Metasystems, Altlussheim).

LSI EGR1(5q31)/D5S23,D5S721(dual color probe)

D5S23,D5S721: hybridize on the short arm of chromosome 5 in band p15.2 with SpectrumGreen, and

EGR-1(early growth response-1): hybridize on the long arm of chromosome 5 in band q31 with SpectrumOrange

LSI D7S486(7q31)/CEP7(dual color probe)

D7S522: hybridize to an anonym locus in the long arm of chromosome 7 in band q31 with SpectrumOrange, and

CEP 7: hybridize to the centromere of chromosome 7 (7p11.1-7q11.1) with SpectrumGreen

2.5 Statistical analysis

Statistical analysis was performed by means of the nonparametric Mann–Whitney test and the 2-way analysis of variance (ANOVA) test using the GraphPad Prism statistical software (San Diego, CA). Grouped data were expressed as means +/- 1 standard deviation.

2.6 Terminology

The whole chromosome set can just be identified in a defined phase of the cell cycle, namely the metaphase. In this phase the chromosomes become aligned but the chromatids are still not separated. Adding colchicine, the cell cycle will be arrested in the metaphase. The description of human chromosomes is following an international consensus, recommended in “International System for Human Cytogenetic Nomenclature) (ISCN 2005)”.

Chromosomes are rated in size, position of the centromere (separating p and q arm of one chromosome), and banding pattern. The autosomes are numbered from 1 to 22, the sex chromosomes are referred to as X and Y.

In the description of a karyotype, first the total number of chromosomes per cell and after the sex chromosome constitution is given. The autosomes are specified only when an abnormality exists. There can be separated numerical (whole chromosome gain [+] or loss [-]) from structural abnormalities. The most common structural abnormalities are translocations, deletions inversions, insertions and isochromosomes.

In the present study the only structural abnormality found were deletions (del). A deletion describes the loss of parts of chromosome material. There are terminal deletions with one breakpoint in the p or q arm: del(5)(q12), and interstitial deletions with two breakpoints in one arm and reunion: del(5)(q15q33). The segment lying between these breakpoints has been deleted.

3. Results

3.1 Reserves, morphologic, and immunophenotypic characteristics of BM MSCs

Our MSCs cultures were successfully expanded in all 13 MDS patients and 20 healthy individuals. And, all expanded MSCs displayed the characteristic spindle-like morphology (figure 7 and 11).

The estimated frequency of MSCs in the BMMC fraction according to the LDA did not differ significantly between MDS patients (21.74 +/- 13.59 per 10^5 BMMCs) and healthy controls (24.84 +/- 15.43 per 10^5 BMMCs; P = 0.7823) presented in figure 9.

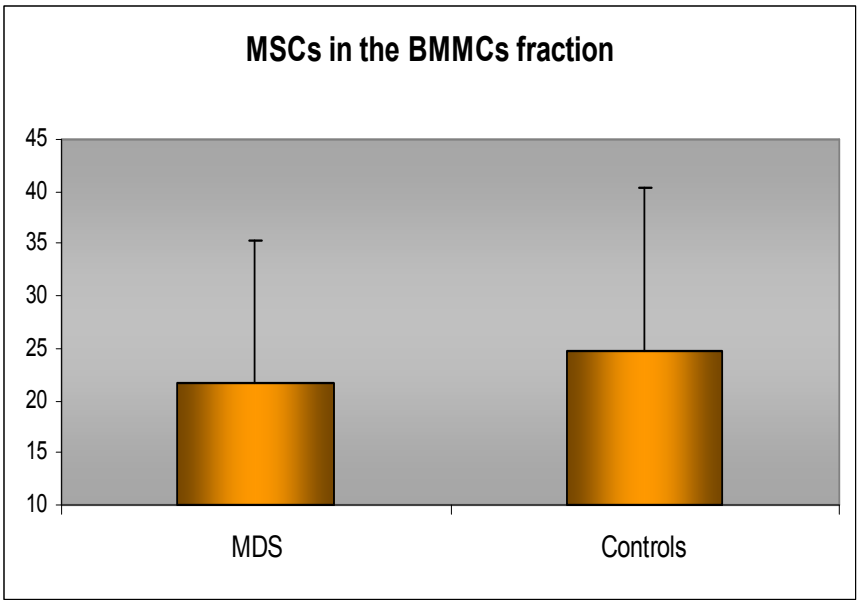


Figure 9. Percentage of MSCs within the BMMCs (bone marrow mononuclear cell) fraction in MDS patients (left) in comparison to controls (right).

The immunophenotypic analysis at the end of each passage demonstrated that cultures constituted of a homogenous cell population positive for CD73, CD90, CD146, CD105, CD29, CD44 surface antigens and negative for the hematopoietic markers CD45, CD14, and CD34 (figure 10).

These data suggest that the reserves, morphologic, and immunophenotypic characteristics of BM MSCs in MDS patients are similar to the normal individuals.

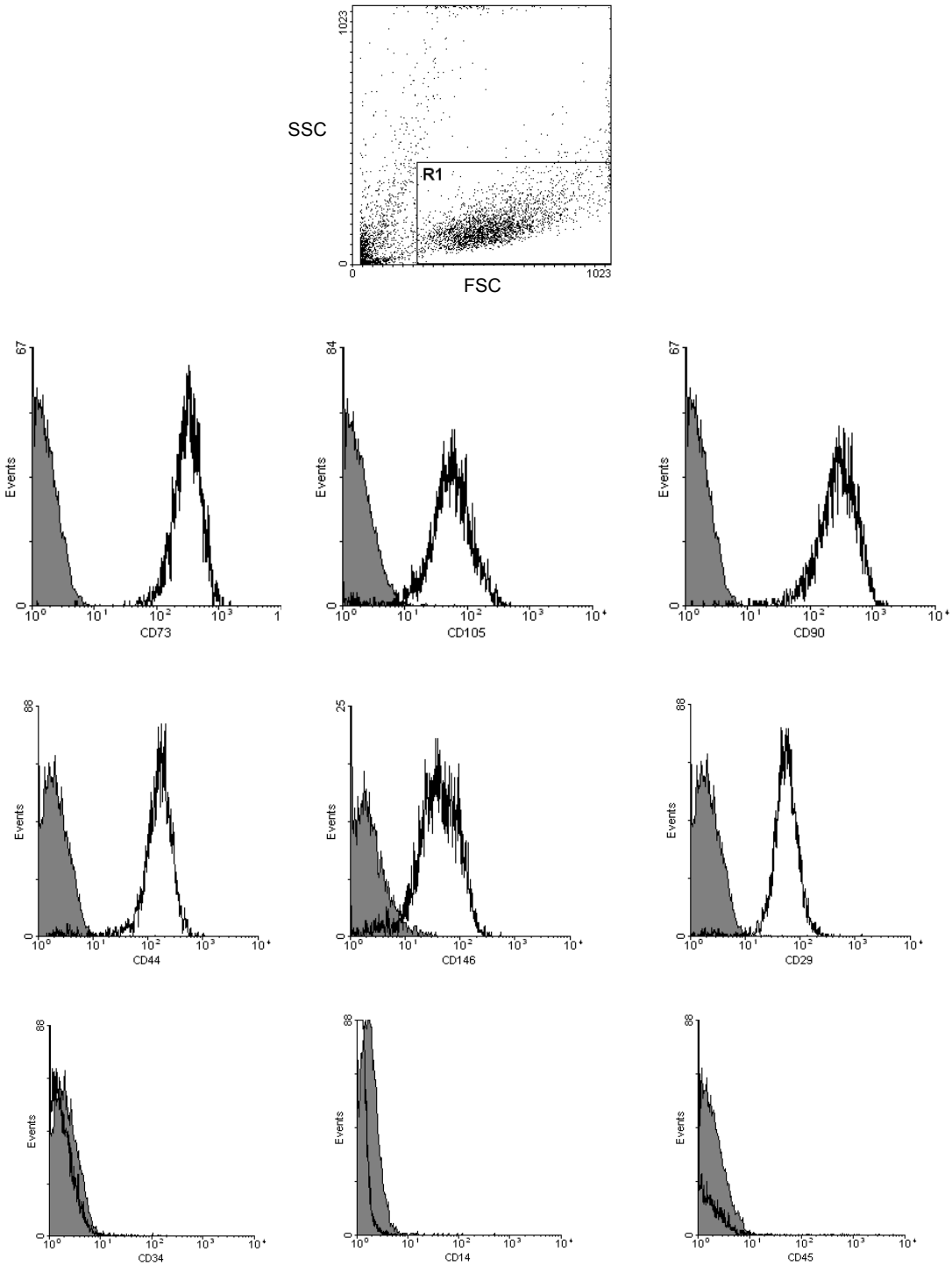


Figure 10. Immunophenotypic characteristics of mesenchymal stem cells (MSCs). The graphs show the flow cytometric characteristics of MSCs at passage 2 from a representative myelodysplastic syndromes (MDS) patient. The forward scatter (FSC) versus side scatter (SSC) dot plot depicts the gate of MSCs population (R1). The open histograms show the expression of positive (CD73, CD105, CD90, CD44, CD146, CD29) and negative (CD34, CD14, CD45) surface markers in comparison to isotype matched controls (gray-filled histograms) in MSCs gated in R1.

3.2 Differentiation potential of MSCs

Culture-expanded MSCs from MDS patients were able to differentiate into adipogenic, osteogenic, and chondrogenic lineages as was shown by the respective cytochemical staining (figure 11). Furthermore, the adipogenic, osteogenic, and chondrogenic differentiating capacity assessed by the relative mRNA expression of aP2 and PPARG, ALP and RUNX2, COL2A1 and AGC1, respectively, did not differ significantly between MDS patients and controls (P = 0.7394 and P = 0.6842, P = 0.8534 and P = 0.5288, P = 0.123 and P = 0.1431, respectively; figures 12+13). These data suggest that BM MSCs from MDS patients display normal differentiation potential.

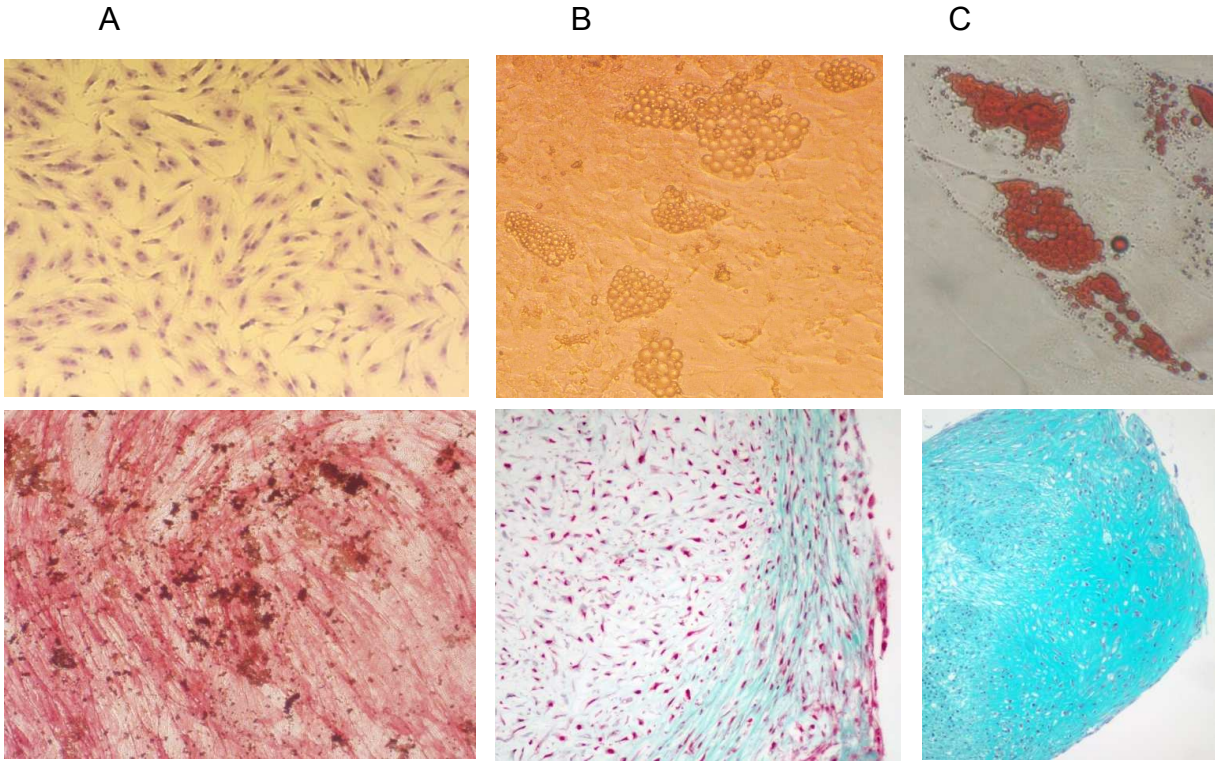


Figure 11. Differentiation potential of mesenchymal stem cells (MSCs). The upper panel shows undifferentiated MSCs from P2 (**A**) and differentiated cells toward the adipogenic (**B** and **C**), osteogenic (**D**), and chondrogenic (**E** and **F**) lineages from a representative myelodysplastic syndromes (MDS) patient. Adipogenenesis was induced upon culture in medium supplemented with 1-methyl-3-butylisoxanthine/dexamethasone/indomethacin/insulin and was assessed by morphologic evaluation of unstained (**B**) and Oil Red O-stained (**C**) cells. Osteogenesis was induced following culture in medium supplemented with dexamethasone/ascorbate-2-phosphate/NaH 2 PO 4 and was assessed by ALP/von Kossa staining (**D**). Chondrogenesis was induced in MSC pellets cultured in the presence of insulin/transferrin/linoleic acid /bovine serum albumin/sodium pyruvate/ascorbate-2-phosphate/dexamethasone/ l -proline/selenous acid/transforming growth factor-β 3 and was assessed by Masson (**E**) and Alcian blue (**F**) staining.

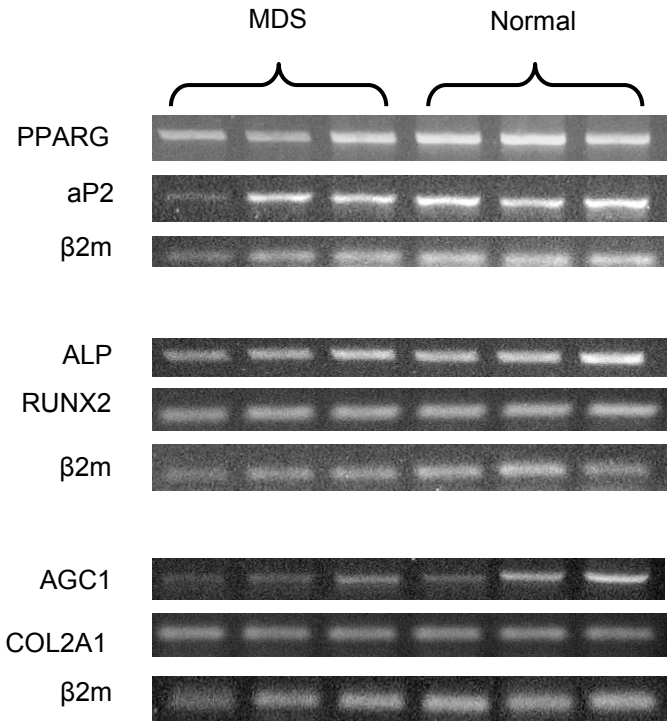


Figure 12. This images depicts specific gene mRNA expression of P2 BM MSCs upon differentiation toward the adipogenic (aP2 and PPARG), osteogenic (ALP and RUNX2), and chondrogenic (COL2A1 and AGC1) lineages in representative MDS (*n* = 3)

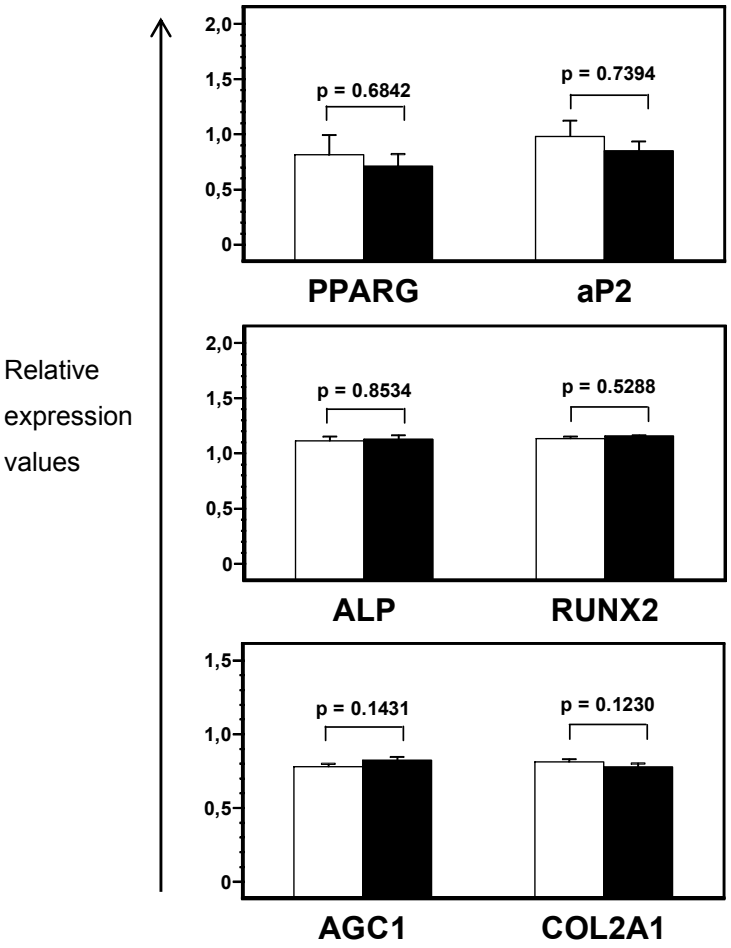


Figure 13. This 3 graphs show the cumulative data (mean relative values +/- SEM) of specific gene mRNA expression from all MDS (■) and controls (□) studied. Comparison between patients and controls has been performed by means of the nonparametric Mann–Whitney test.

Abbreviations: AGC1, aggrecan; ALP, alkaline phosphate; aP2, adipose fatty acid-binding protein; COL2A1, collagen type II; PPARG, peroxisome proliferator-activated receptor-γ; RUNX2, runt-

3.3 Clonogenic and proliferative potential of MSCs

Data showing the clonogenic and proliferative potential of MSCs in MDS patients and healthy controls are presented in figure 14. CFU-F recovery by culture-expanded MSCs was significantly lower in MDS patients compared to controls through P1–P6 culture period (F = 14.359, P < 0.01) suggesting defective clonogenic capacity of patient MSCs. The doubling time of MSCs during the P1–P6 culture period was significantly increased in MDS patients compared to controls (F = 67.58405, P < 0.001). Specifically, the MSC doubling time ranged from 4.62 +/- 1.85 days (P1) to 9.28 +/- 2.71 days (P6) in patients and from 2.31 +/- 0.87 days (P1) to 5.06 +/- 1.03 days (P6) in controls. In accordance with the cell doubling time data were the results from MTT assay at a representative passage (P2). The number of live cells corresponding to the obtained optical density gradually increased over the 13-day culture period, however, remained significantly lower in MDS patients compared to controls (F = 69.3220, P < 0.001) corroborating further the defective proliferative potential of MSCs in MDS.

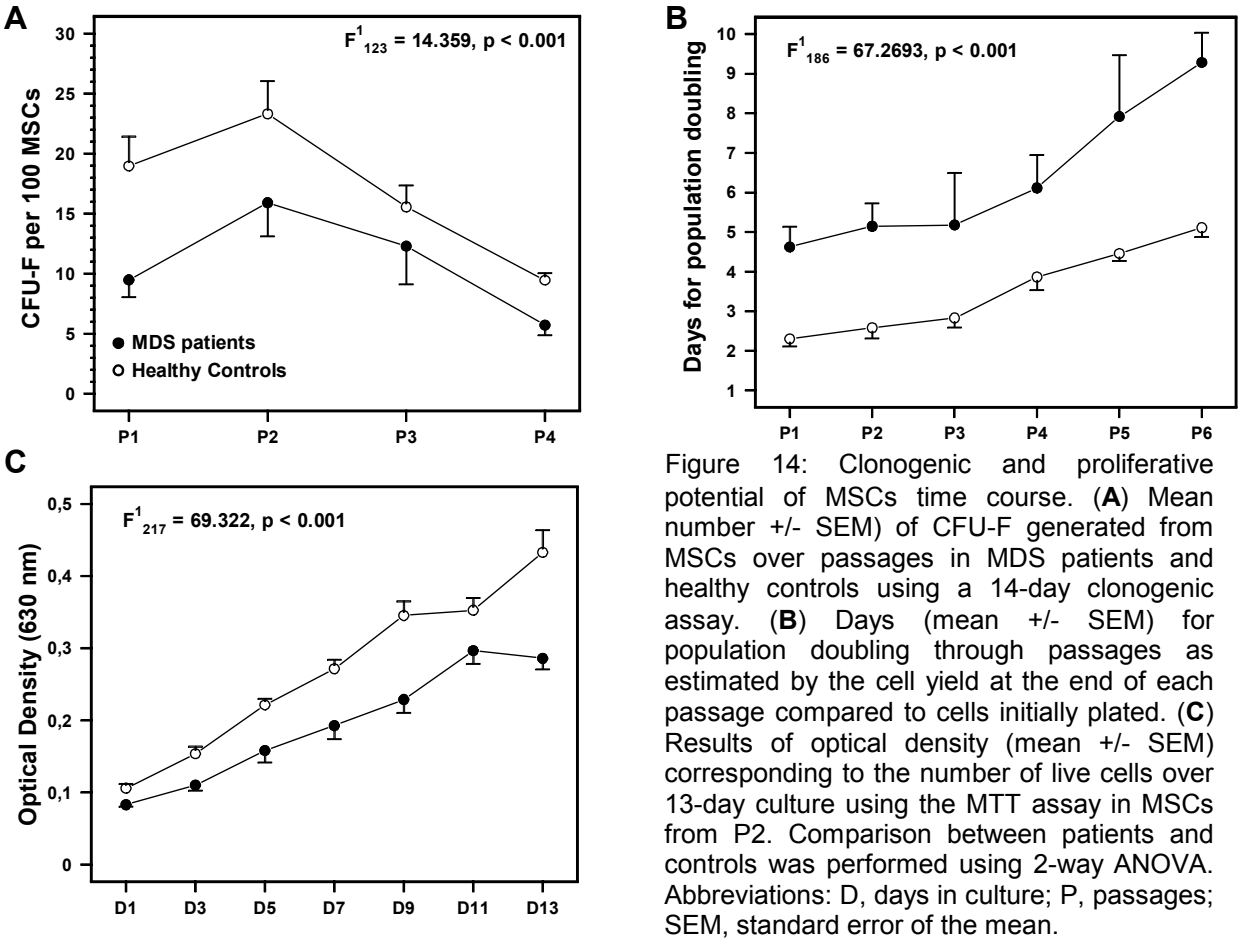


Figure 14: Clonogenic and proliferative potential of MSCs time course. (**A**) Mean number +/- SEM) of CFU-F generated from MSCs over passages in MDS patients and healthy controls using a 14-day clonogenic assay. (**B**) Days (mean +/- SEM) for population doubling through passages as estimated by the cell yield at the end of each passage compared to cells initially plated. (**C**) Results of optical density (mean +/- SEM) corresponding to the number of live cells over 13-day culture using the MTT assay in MSCs from P2. Comparison between patients and controls was performed using 2-way ANOVA. Abbreviations: D, days in culture; P, passages; SEM, standard error of the mean.

3.4 Immunosuppressive properties of MSCs

To determine whether patient MSCs could efficiently inhibit T-cell proliferative responses, we mixed normal or patient MSCs with allogeneic normal T lymphocytes in the presence of PHA or IL-2 and we measured ^{3}H-TdR incorporation. As shown in figure 15, a significant reduction was obtained in T-cell proliferation when cultures of purified T lymphocytes stimulated by PHA or IL-2 (3,377 +/- 1,674 cpm and 16,934 +/- 24,777 cpm, respectively) were performed in the presence of allogeneic normal MSCs (302 +/- 99 cpm and 1,753 +/- 1,734 cpm, respectively; *P* = 0.0034 and *P* = 0.0317, respectively). Similarly, T-cell proliferative responses to the above activators (21,608 +/- 22,610 cpm and 56,211 +/- 41,093 cpm, respectively) were significantly reduced in the presence of MSCs from MDS patients (657 +/- 281 cpm and 10,561 +/- 13,733 cpm, respectively; *P* = 0.0079 and *P* = 0.0463, respectively). The percentage of inhibition of PHA-induced or IL-2-induced T-cell proliferation by MSCs did not differ significantly between patients (90.59% +/- 8.04% and 84.14% +/- 10.68%) and controls (88.53% +/- 6.45% and 82.39% +/- 12.25%, respectively; *P* = 0.6905 and *P* = 1.0, respectively). Taken together these data suggest that MSCs from MDS patients display normal immunosuppressive properties in terms of the capacity to inhibit T-cell proliferation induced by mitogens.

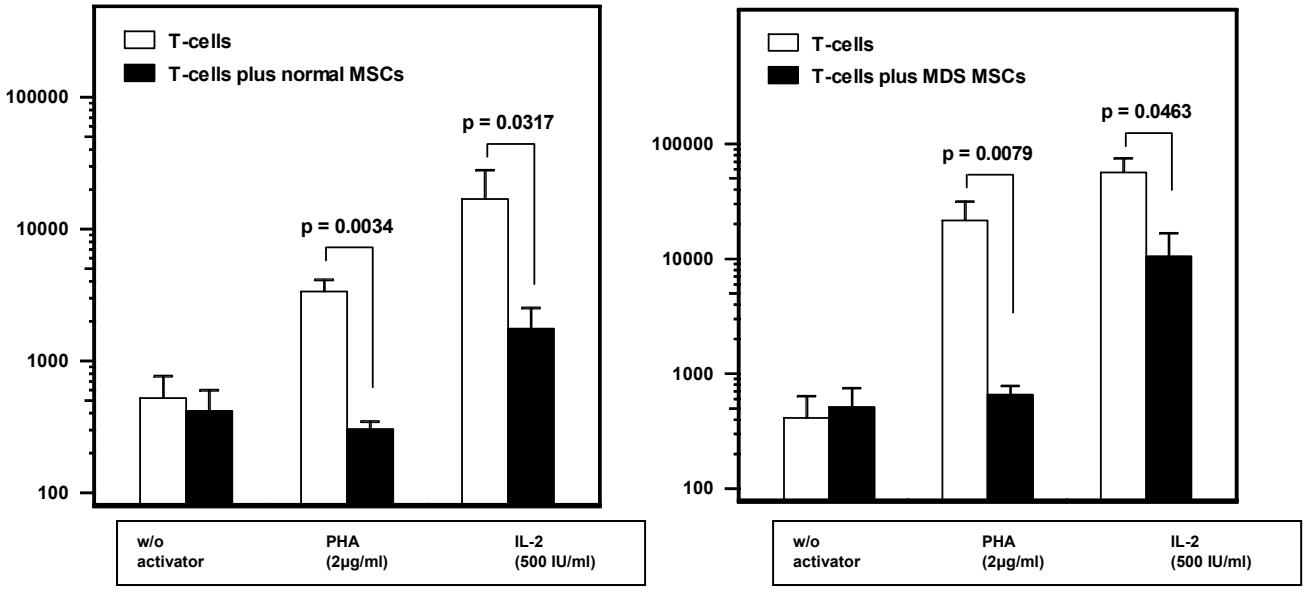


Figure 15. Inhibitory effect of mesenchymal stem cells (MSCs) on T-lymphocyte proliferation. The bars represent the proliferative responses (expressed in counts per minute upon 3 H-TdR incorporation) of unstimulated and activator-induced T lymphocytes in the presence or absence of BM MSCs from allogeneic normal (left panel) or patient (right panel) MSCs. Data are expressed as mean (+/-SEM) of triplicates of 5 separate experiments. Comparison of counts per minute in the presence or absence of MSCs was performed by means of the nonparametric Mann–Whitney test and the *P* values indicating the statistical significance are shown.

3.5 Cytokine production by MSCs

To investigate whether BM MSCs are the primary source of the proinflammatory cytokines known to be associated with the pathophysiology of MDS, we assessed TNF-α, IL-1β, and IL-6 levels in culture supernatants from P2, P4, and P6. No statistically significant difference was found between patients and controls in the levels of cytokines time course. Furthermore, the levels of the growth-promoting cytokines VEGF and SDF-1α during the P2–P6 culture period did not differ significantly between patients and controls (table 6, figure 16) suggesting that abnormal cytokine production by MSCs per se seems unlikely to be the cause of the impaired clonogenic and replicative capacity of MSCs in MDS.

Table 6. Cytokine Levels in the Supernatants of MSC Cultures Time Course.

	MDS patients (n = 13)			*Healthy controls (n = 20)*			
	P2	*P4*	*P6*	*P2*	*P4*	*P6*	*P value*[a]
TNF-α (pg/mL)	0.24 ± 0.03	0.23 ± 0.02	0.23 ± 0.05	0.22 ± 0.08	0.28 ± 0.20	0.23 ± 0.03	N.S.
IL-1β (pg/mL)	0.95 ± 3.22	0.62 ± 1.19	0.92 ± 1.06	2.54 ± 2.30	0.23 ± 0.34	0.78 ± 0.69	N.S.
IL-6 (ng/mL)	6,521 ± 3,604	6,481 ± 2,863	6,189 ± 3,636	5,061 ± 2,116	7,514 ± 3,295	5,443 ± 3,349	N.S.
VEGF (pg/mL)	2,401 ± 872	1,683 ± 761	2,063 ± 798	2,035 ± 791	1,711 ± 1,095	1,711 ± 602	N.S.
SDF-1α (pg/mL)	3,435 ± 2,312	3,375 ± 1,711	2,959 ± 1,097	3,842 ± 1,642	3,193 ± 1,339	2,476 ± 1,133	N.S.

Measurements have been performed by means of an enzyme-linked immunosorbent assay. Cytokine levels between MDS patients and healthy controls time course were performed by 2-way ANOVA. Abbreviations: IL, Interleukin; MDS, myelodysplastic syndromes; MSCs, mesenchymal stem cells; N.S. non-significant difference; P, passage; SDF-1α, stromal-derived factor-1α; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

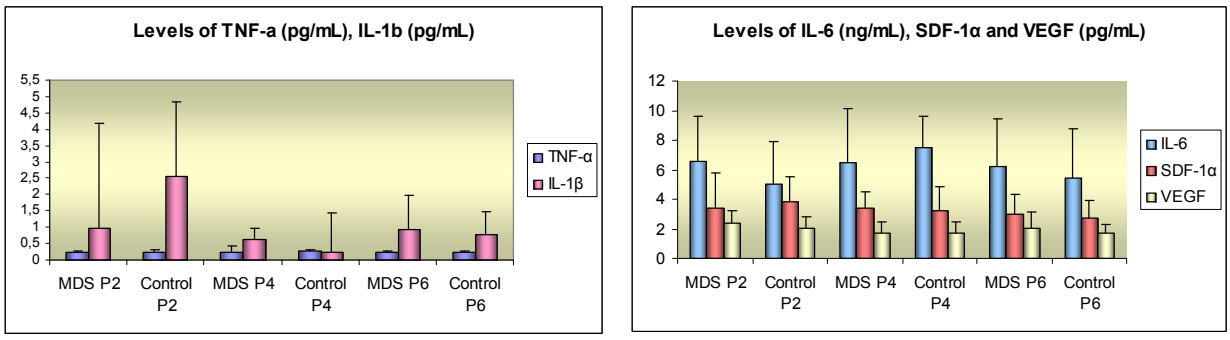


Figure 16. Cytokine production from MDS MSCs compared to normal MSCs in passages 2, 4, and 6.

3.6 Cytogenetic analysis of BM hematopoietic cells and MSCs

Cytogenetic analysis in MDS patients

Results from the cytogenetic analysis of patient BM hematopoietic cells and MSCs are shown in table 7. In total, karyotype abnormalities were identified in BM hematopoietic cells in 8 out of 13 MDS patients. Specifically, 5 patients displayed interstitial deletion of the long arm of chromosome 5 [del(5q)] with an additional del(7q) subclone in 1 patient, 2 patients had trisomy 8 (+8), and 1 patient −Y. The chromosomal analysis of the corresponding MSCs did not show any of these abnormalities at P2–P8. The presence of del(5q) and trisomy 8 in hematopoietic cells and their absence in MSCs was verified by FISH analysis. Interestingly, cultures of patient MSCs showed a clonal trisomy 5 in 3 cases and trisomy 7 in one case. Although the trisomy 5 was identified in only 1 out of 4 passages (P4 in one case and P6 in 2 cases) with classical cytogenetics, FISH analysis revealed the existence of the trisomy 5 in all passages analyzed (P2, P4, P6, P8; table 8). Trisomy 7 was identified only in the only passage tested (P2) and was confirmed by FISH analysis. The absence of trisomies 5 and 7 in the corresponding hematopoietic cell cultures was confirmed by FISH analysis.

Table 7. Clinical and cytogenetic data of BM hematopoietic cells and MSCs of MDS patients.

No	Age	WHO subtype	IPSS risk category	Karyotype BM Hematopoietic Cells	MSC passage	Karyotype BM MSCs
Pt-1	65	RAEB-2	High	46,XX,del(5)(q15q33)[8]/ 46,XX,del(5)(q15q33),del(7)(q22)[4]	2	
					4	46,XX[25] 47,XX,+5[2]/46,XX[19]
					6	46,XX[11]
					8	46,XX[10]
Pt-2	80	RAEB-1	Intermediate -1	46,XX,del(5)(q15q33)[15]	2	
					4	no metaphases found 46,XX[15]
					6	47,XX,+5[3]/46,XX[4]
					8	46,XX[1]
Pt-3	81	RA	Low	46,XX,del(5)(q12q33)[15]	2	
					4	no metaphases found 46,XX[20]
					6	47,XX,+5[2]/46,XX[10]
					8	46,XX[9]
Pt-4	62	RAEB-1	Intermediate -1	46,XY,del(5)(q13q33)[21]/46,XY[1]	2	no metaphases found
					4	46,XY[8]
					6	46,XY[4]
					8	46,XY[2]
Pt-5	67	RA	Low	46,XX,del(5)(q13q33)[14]/46,XX[6]	2	46,XX[20]
Pt-6	66	RA	Intermediate -1	47,XY,+8[3]/46,XY[2]	2	no metaphases found
					4	46,XY[20]
					6	46,XY[6]
					8	46,XY[15]
Pt-7	75	RA	Intermediate -1	47,XY,+8[9]/46,XY[11]	2	46,XY[20]
Pt-8	80	RA	Low	45,X,-Y[4]/46,XY[14]	2	46,XY[23]
Pt-9	62	RAEB-2	Intermediate -2	46,XX[20]	2	46,XX[4]
					4	46,XX[1]
					6	46,XX[13]
					8	46,XX[10]
Pt-10	70	RAEB-1	Intermediate -2	46,XY[20]	2	47,XY,+7[7]/46,XY[33]
Pt-11	60	RA	Low	46,XX[20]	2	46,XX[25]
Pt-12	84	RAEB-2	Intermediate -2	46,XX[25]	2	46,XX[18]
Pt-13	83	RAEB-2	Intermediate -2	46,XY[20]	2	46,XY[20]

Abbreviations: BM, Bone Marrow; MSCs, Mesenchymal Stem Cells; MDS, Myelodysplastic syndrome; No, Number; Pt, Patient; WHO, World Health Organization; IPSS, International Prognostic Scoring System; RA, Refractory Anemia; RAEB, RA with Excess of Blasts.

Table 8. FISH analysis from MSC cultures showing karyotypic abnormalities.

No	FISH marker	MSC passage	% of aberrant BM MSCs
Pt-1	+5	2	46%
		4	47%
		6	21%
		8	2%
Pt-2	+5	2	70%
		4	70%
		6	62%
		8	60%
Pt-3	+5	2	10%
		4	6%
		6	65%
		8	4%
Pt-10	+7	2	26%
ND-1	+5	2	38%

Abbreviations: FISH, Fluorescence In Situ Hybridization; BM, Bone Marrow; MSCs, Mesenchymal Stem Cells; No, Number; Pt, Patient; ND, Normal Donor.

Cytogenetic analysis of healthy subjects

As anticipated, chromosome analysis of BM hematopoietic cells from healthy individuals showed normal karyotype (table 9). The chromosome analysis of the respective MSCs, however, showed a clonal trisomy 5 in one case at P2 (figure 17a+b). Pre-existence of trisomy 5 in BM hematopoietic cells of this subject was excluded by FISH. Because trisomies 5 and 7 were only identified in a small number of analyzed metaphases and in only 1 out of 4 passages in 4 patient and one control MSC cultures, while a FISH screening revealed the existence of trisomy 5 or 7 in interphase cells of all passages, an additional FISH screening with probes for chromosomes 5 and 7 was performed in all the remaining cases with normal MSC karyotype (healthy and patients MSCs) in all passages. No further cases with a masked trisomy 5 or 7 were identified. Taken together, these data show that although BM MSCs from MDS patients do not show the cytogenetic abnormalities present in BM hematopoietic cells, they may show irrelevant clonal abnormalities during passages. The pathophysiologic significance of this abnormality, however, is questionable because a similar abnormality was also identified in normal MSCs.

Table 9. Cytogenetic data of normal BM hematopoietic cells and MSCs

No	Age	Karyotype BM Hematopoietic Cells	MSC passage	Karyotype BM MSCs
ND-1	80	46,XY[20]	2	47,XY,+5[5]/46,XY[29]
ND-2	62	46,XY[25]	2	46,XY[5]
ND-3	75	46,XY[23]	2	46,XY[20]
ND-4	70	46,XY[20]	2	46,XY[15]
ND-5	83	46,XY[20]	2	46,XY[5]

Abbreviations: BM, bone marrow; MSCs, Mesenchymal Stem Cells; No, Number; ND, Normal Donor.

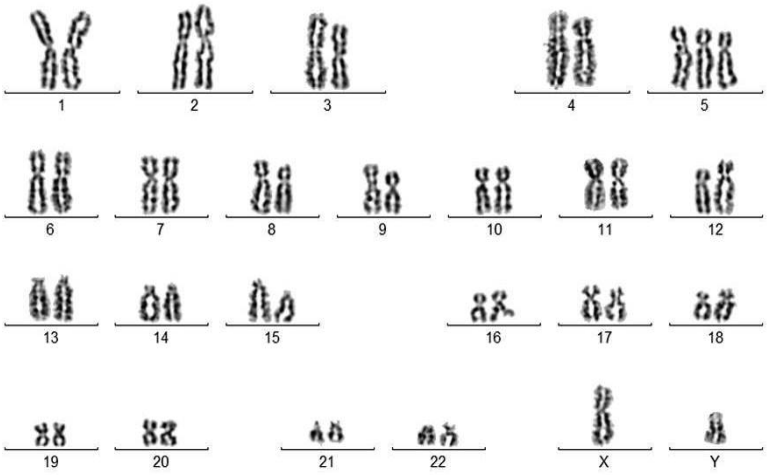


Figure 17a. Cytogenetic analysis of mesenchymal stem cells (MSCs) derived from the normal donor-1 at P2. Chromosome banding analysis showing trisomy 5.

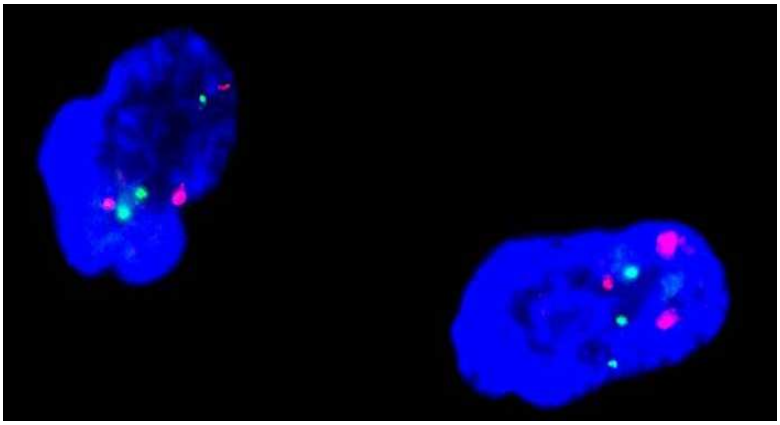


Figure 17b. FISH analysis with a dual color probe LSI EGR1 (Spectrum Orange 5q31)/ D5S23,D5S721 (Spectrum Green, 5p15.2) showing 3 signals each on 2 interphase nuclei from MSCs.

4. Discussion

A still un-clarified issue are the mechanisms leading to hematopoietic failure in patients with MDS, however, there is evidence suggesting that not only intrinsic abnormalities within the HSC compartment but also defectives in the bone marrow microenvironment may play a role ([82]). The existing data on this field of research are limited and contradictory.

In this study, we have evaluated the quantitative, functional, and cytogenetic characteristics of BM MSCs in patients with *de novo* MDS in order to explore whether the BM stromal cells are primarily affected in these patients.

MSCs from all MDS patients studied were expanded successfully and were morphologically and immunophenotypically indistinguishable from the respective of the healthy controls. These observations are in agreement with data of Flores-Figueroa et al. also showing normal development and immunophenotypic characteristics of MDS-derived MSCs ([63]) whereas are in contrast with data of Lopez-Villar et al. suggesting that MDS MSCs occasionally fail to grow *in vitro* ([65]). These inconsistencies might be attributed to the heterogeneity of MDS patients used in these studies in terms of WHO type and IPSS risk category.

Despite the successful expansion of patient MSCs, we found that the rate of cellular growth through passages, assessed by the population doubling time, was significantly reduced in MDS patients compared to controls, suggesting defective proliferative capacity of MSCs. This finding was further substantiated by the MTT assay that also showed low rate of cell proliferation of MDS derived MSCs in comparison to normal subjects as well as by the CFU-F clonogenic assay that revealed defective colony formation of patient MSCs through passages compared to normal MSCs. Defective proliferation is revealed by an overall prolonged in vitro expansion time for patient cells, or even in cases by expansion-inability. The proliferation deficiency is not attributed to increased cell-apoptosis, but rather to cell-senescence; this latter is proposed by either a defective time-course clonogenic potential during in vitro expansion, or even by an earlier cell-expansion arrest. One possible explanation for early cell-senescence in patient cells could be the previous exposure of cells to long-term inflammatory BM microenvironment (with increased

TNFα levels), as it is reported for MSCs in other inflammatory disease ([75]). Or, these abnormalities might represent a primary MSC defect.

Using a LDA, we found that the frequency of BM MSCs within the BMMC fraction was similar in patients and healthy controls suggesting normal reserves of MSCs in MDS BM.

Regarding the MSC differentiation capacity, we showed that culture-expanded MSCs from the patients displayed normal osteogenic, chondrogenic, and adipogenic potential. Previous studies have suggested impaired chondrogenic or adipogenic potential of BM MSCs in MDS based mainly on the morphologic evaluation of immunohistochemically stained differentiated cells ([26], [65]). However, in our study we have evaluated the differentiation potential of MSCs based not only on the assessment of the morphological and cytochemical characteristics of cells upon differentiation but also on the quantification of differentiation-related specific gene mRNA expression. Normal differentiation potential of MDS-derived BM MSCs has been also proposed by others based on cytochemical findings ([63]).

Normally MSCs possess immunosuppressive properties and inhibit T-cell proliferation to alloantigens and mitogens ([46]). In some MDS patients with marrow hypocellularity, pathogenesis is associated with aberrant immune responses and T-cell mediated inhibition of hemopoiesis ([25]). Do patient MSCs possess normal immunosuppressive properties, and could they be safely used for autologous therapeutic purposes in MDS patients? In order to answer these issues, the capacity of MDS BM MSCs to suppress T-cell immune responses *in vitro* was evaluated. In contrast to previous reported data suggesting impaired immunosuppressive potential of MDS derived BM MSCs ([62]), [64]), we found that patient MSCs sufficiently inhibited T-cell proliferation induced by mitogens. The discrepancy might be attributed to differences in the methodology. For example, in a report where MDS-RA primordial MSCs ($Flk1^+/CD31^-/CD34^-$) were studied, the defective immunosuppressive capacity against mitogen-activated T-cell proliferation was confirmed by MSC failure to block T-cells in G0/G1-phase, and concomitant MSC failure to inhibit specific T-cell activation markers ([62]). MDS-RA primordial MSCs under-expressed TGFβ1, a major MSC-derived immunoregulatory molecule. This finding could explain the observed reduced suppression on T-cell proliferation. However, our data suggest that BM MSCs is unlikely to have a major role in the aberrant T-cell responses occasionally seen in MDS patients.

In accordance with this assumption was the normal production of TNF-α, IL-1β, and IL-6 by MSC stromal layers time course suggesting that the BM MSCs *per se* are not the main cellular source of these proinflammatory cytokines in patients' BM. Similarly, the levels of the growth-promoting cytokines VEGF and SDF-1α in patient MSC culture supernatants were within normal range time course. Also another group has reported, that intrinsic MSC cytokine production (TNFα, IL-1β, IL-6, SCF, GM-CSF, and VEGF) and extracellular matrix deposition (fibronectin and collagen) are within the normal limits in patient cells ([63]), implying that MSC cytokine production and matrix deposition *per se*, are not responsible for the abnormal patient hemopoiesis.

The chromosome-banding analysis of BM hematopoietic cells revealed clonal abnormalities in 8 out of 13 MDS patients and, as expected, normal karyotype in 5 healthy controls studied. Chromosomal analysis of the corresponding MSC cultures showed karyotype abnormalities in 4 out of 13 MDS patients (trisomies 5 and 7) and in 1 out of 5 healthy individuals (trisomy 5) but different from those found in the BM hematopoietic cells. These data indicate that MSC cultures from MDS patients do not show the cytogenetic abnormalities present in the BM hematopoietic cells at diagnosis and, accordingly, they do not belong to the abnormal clone.

Since the cytogenetic characterization of BM derived MSCs from MDS patients, reported in only a few studies, is rather controversial, also our study data can not contribute to a final consensus so far as these studies suggesting a) that MDS-derived MSCs do not harbour the cytogenetic aberrations present in the corresponding BM hematopoietic cells ([66], [27]) and b) that MDS MSCs show clonal cytogenetic abnormalities ([61], [67], [65]). One explanation, the available data on the cytogenetic analysis of MSCs from MDS patients are controversial probably due to the methodology diversities. Furthermore, different groups have shown a big variety in the kind of aberration reported, that is hypodiploidy, balanced translocations, whole chromosome gains, and deletions. Most interestingly, all of the cytogenetic aberrations described in patient MSCs are non-associated with the aberrations found in the corresponding BM HCs. Notably in addition, most of the reported aberrations in BM MSCs are non-characteristic for MDS ([83]). Moreover, cytogenetic aberrations in MSCs have also been identified in MDS cases with normal HC karyotype, and even in MSCs derived from normal donors, implying that the observed aberrations could be attributed to the in vitro cell expansion process.

Since the existing data are limited, with only few cases considered, more future studies are needed. Divergence on MSC cytogenetic profile could be explained by the aforementioned non-separation of patients to specific MDS sub-types and also by the different resolutions of methodologies used by different research groups (listed in table 5). Nevertheless overall data favour the conception of patients' predisposition to genetic instability.

Additionally, we have shown that patient MSC cultures displayed chromosomal stability until late passages. This finding is in accordance with results from other groups reporting normal karyotype until late MSC passages from patients with different hematologic disorders including MDS and healthy individuals ([84], [85]). The trisomy 5, found in MSC cultures from 3 MDS patients and one healthy control, has been described in acute lymphoblastic and myeloid leukemias as well as in neoplastic and non-neoplastic solid tumors ([86], [87], [88], [89], [http://AtlasGeneticsOncology.org/Anomalies/tri5ID1255.html). Trisomy 5 has also been reported as a probable nonrandom phenomenon in long-term cultures from solid tumor patients ([90]). Because trisomy 5 was identified in both patient and normal MSC cultures and chromosome gains have already been described in cell cultures of normal tissues [[91], [92]), we assume that the abnormality probably represents a non-random in vitro phenomenon with presently unknown significance. Trisomy 7, found in MSC cultures from one MDS patient, has not been described in hematologic diseases (http://atlasgeneticsoncology.org/Indexbychrom/idxa_7.html) but it is a common finding in malignant and non-malignant tissues ([93], [94], [95]). It has been suggested, however, that trisomy 7 accumulates with age and may be associated with age progression rather than with a disease state ([93]). Accordingly, the pathophysiologic significance of this abnormality in patient's MSC cultures remains uncertain.

In conclusion, data from this study show that BM MSCs from patients with de novo MDS are normal in regards to the number, the differentiation potential, and immunosuppressive properties. Patient MSCs do not show any aberrations in the production of proinflammatory or growth-promoting cytokines and do not harbour the cytogenetic abnormalities present in hematopoietic cells. Patient MSC cultures display chromosomal stability time course, however, they may develop irrelevant chromosomal alterations with unknown pathophysiologic significance as they may also occur in MSC cultures from normal individuals. Finally, MDS-derived MSCs

display impaired proliferative and clonogenic potential probably associated with the chronic inflammatory process present in patients' BM. Overall, our data suggest that BM MSCs from MDS patients do not belong to the abnormal clone and do not display major quantitative and functional abnormalities. The underlying mechanism for the impaired proliferative/clonogenic potential and the pathophysiologic significance of the subtle chromosomal alteration occurred during culture is an interesting field for further investigation.

Larger future studies are needed and it is essential to analyze patient data by categorizing patients in MDS subtypes in order to make more safe conclusions. Application of new technologies (such as microarray gene expression, or array-CGH), will help to elucidate the true MSC genetic profile.

5. References

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ORIGINAL RESEARCH REPORT

Reserves, Functional, Immunoregulatory, and Cytogenetic Properties of Bone Marrow Mesenchymal Stem Cells in Patients With Myelodysplastic Syndromes

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Defective hematopoiesis supporting capacity of bone marrow (BM) stroma has been implicated in the pathophysiology of myelodysplastic syndromes (MDS). The aim of this study is to explore whether the BM stroma progenitors, namely the mesenchymal stem cells (MSCs), are primarily affected in MDS by evaluating the reserves, the functional properties, as well as the cytogenetic characteristics, in comparison to BM hematopoietic cells, in patients with de novo MDS ($n = 13$). The number, differentiation potential toward adipocytes/chondrocytes/osteoblasts and immunosuppressive function in terms of inhibition of mitogen-induced T-cell proliferation did not differ significantly between patient and normal ($n = 20$) MSCs. Patient MSCs did not show any aberrations in the production of proinflammatory or growth-promoting cytokines and did not harbor the cytogenetic abnormalities present in hematopoietic cells. Occasional patient and normal MSC cultures, however, developed irrelevant chromosomal alterations (trisomies 5 and 7) with uncertain pathophysiologic significance. Compared to controls, patient MSCs displayed impaired proliferative and clonogenic potential through passages that might represent a nonspecific abnormality associated with the chronic inflammatory process present in patients' BM. These data suggest that BM MSCs from MDS patients do not belong to the abnormal clone and do not represent the main cellular source contributing to the inflammatory marrow microenvironment.

Introduction

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal hematopoietic stem cell (HSC) malignancies characterized by ineffective bone marrow (BM) hematopoiesis, peripheral blood (PB) cytopenias, and substantial risk for progression to acute myeloid leukemia [1–3]. Abnormal hematopoiesis in MDS is considered to be the result of a multistep process implicating genetic, epigenetic, and immune-mediated alterations of an early HSC resulting in excessive apoptosis of its marrow progeny [4]. Excessive apoptosis, however, the hallmark of ineffective hematopoiesis, is not restricted to MDS clone but affects also the normal hematopoietic cells, suggesting a possible apoptosis-inducible effect of the marrow microenvironment [5–7]. This might be due to abnormal hematopoietic-to-stromal cell interactions [8], presence of activated clone-directed lymphocytes and macrophages affecting both normal and clonal BM subpopulations [9,10], relative deficiency of hematopoietic growth factors or aberrant release of inhibitors [7,11,12].

The hematopoiesis supportive cellular elements of BM stroma, namely the adipocytes, osteoblasts, and fibroblast-like cells, derive from a common progenitor cell, the mesenchymal stem cell (MSC) [13–15]. In addition to their capacity to support hematopoiesis, MSCs also display local and systemic immunoregulatory and immunosuppressive properties [16–18]. Since a common stem cell with wide differentiation potential, namely the multipotent adult progenitor cell (MAPC), has been proposed for BM HSCs and MSCs [19], it seems reasonable to assume that BM MSCs in MDS patients might belong to the abnormal clone and might be primarily defective. Alternatively, BM MSCs in MDS patients might display secondary genetic aberrations and/or functional defects due to the deranged release of proinflammatory mediators and inhibitory cytokines by the apoptotic

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hematopoietic cells and the activated cellular components of the BM microenvironment.

The possible involvement of BM MSCs in the pathogenetic/pathophysiologic process of MDS has not been extensively studied whereas existing data on the cytogenetic and functional integrity of BM MSCs in MDS patients have shown contradictory results [20–26]. In the current study, we have investigated the reserves and qualitative characteristics of BM MSCs in MDS patients in terms of the proliferative and clonogenic potential, the differentiation capacity, and immunoregulatory properties. The cytogenetic characteristics of BM MSCs in comparison to BM hematopoietic cells as well as the chromosomal stability of BM MSCs during passages have been also studied.

Patients and Methods

Patients

Thirteen patients with de novo MDS and 20 age- and sex-matched healthy individuals, were studied. Patients were classified according to the World Health Organization (WHO) and the International Prognostic Scoring System (IPSS) and were studied on diagnosis before receiving any medication [27,28]. Patient characteristics are summarized in Table 1. The study has approved by the Ethics Committee of the University Hospital of Heraklion and informed consent according to the Helsinki Protocol was obtained from all subjects.

MSC cultures

BM cells from posterior iliac crest aspirates were diluted 1:1 in Dulbecco's modified Eagle's medium-Low Glucose (DMEM-LG; Gibco Invitrogen, Paisley, Scotland) supplemented with 100 IU/mL penicillin–streptomycin (PS; Gibco) and 10 IU/mL preservative-free heparin (Sigma, Saint Louis, MO). The BM mononuclear cells (BMMCs) were obtained following centrifugation on Histopaque-1077 (Sigma) and were cultured in DMEM-LG/10% fetal calf serum (FCS; Hyclone, Logan, UT)/100 IU/mL PS (MSC medium) at a concentration

Table 1. Clinical and Cytogenetic Data of BM Hematopoietic Cells and MSCs of MDS Patients

No	Age	WHO subtype	IPSS risk category	Karyotype BM Hematopoietic Cells	MSC passage	Karyotype BM MSCs
Pt-1	65	RAEB-2	High	46,XX,del(5)(q15q33) [8]/46,XX,del(5)(q15q33),del(7)(q22) [4]	2	46,XX [25]
					4	47,XX,+5 [2]/46,XX [19]
					6	46,XX [11]
					8	46,XX [10]
Pt-2	80	RAEB-1	Intermediate-1	46,XX,del(5)(q15q33) [15]	2	No metaphases found
					4	46,XX [15]
					6	47,XX,+5 [3]/46,XX [4]
					8	46,XX [1]
Pt-3	81	RA	Low	46,XX,del(5)(q12q33) [15]	2	No metaphases found
					4	46,XX [20]
					6	47,XX,+5 [2]/46,XX [10]
					8	46,XX [9]
Pt-4	62	RAEB-1	Intermediate-1	46,XY,del(5)(q13q33) [21]/46,XY [1]	2	No metaphases found
					4	46,XY [8]
					6	46,XY [4]
					8	46,XY [2]
Pt-5	67	RA	Low	46,XX,del(5)(q13q33) [14]/46,XX [6]	2	46,XX [20]
Pt-6	66	RA	Intermediate-1	47,XY,+8 [3]/46,XY [2]	2	No metaphases found
					4	46,XY [20]
					6	46,XY [6]
					8	46,XY [15]
Pt-7	75	RA	Intermediate-1	47,XY,+8 [9]/46,XY [11]	2	46,XY [20]
Pt-8	80	RA	Low	45,X,−Y [4]/46,XY [14]	2	46,XY [23]
Pt-9	62	RAEB-2	Intermediate-2	46,XX [20]	2	46,XX [4]
					4	46,XX [1]
					6	46,XX [13]
					8	46,XX [10]
Pt-10	70	RAEB-1	Intermediate-2	46,XY [20]	2	47,XY,+7 [7]/46,XY [33]
Pt-11	60	RA	Low	46,XX [20]	2	46,XX [25]
Pt-12	84	RAEB-2	Intermediate-2	46,XX [25]	2	46,XX [18]
Pt-13	83	RAEB-2	Intermediate-2	46,XY [20]	2	46,XY [20]

Abbreviations: BM, Bone marrow; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome; MSCs, mesenchymal stem cells; No, number; Pt, patient; RA, refractory anemia; RAEB, RA with excess of blasts; WHO, World Health Organization.

of 2×10^5 cells/cm^2 in 25-cm^2 culture flasks at 37°C/5%CO_2 fully humidified atmosphere. MSC cultures were expanded as previously described [29]. On 70%–90% confluence, cells were detached using 0.25% trypsin–1 mM EDTA (Gibco) and re-seeded for a total of 8 passages (P). Cell-free supernatants were stored at −70°C for measurement of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, stromal-derived factor (SDF)-1α, and vascular endothelial growth factor (VEGF) levels by an enzyme-linked immunosorbent assay (ELISA). All ELISA kits were purchased from R&D Systems (Quantikine, Minneapolis, MN) except the TNF-α kit (Biosource International Inc., Camarillo, CA).

MSC differentiation assays

Trypsinized MSCs from P2 were induced for adipogenic, osteogenic, and chondrogenic differentiation [30,31]. Adipogenic differentiation was induced following 21-day culture of cells in MSC medium supplemented with 10% FCS/0.5 mM 1-methyl-3-butylisoxanthine/1 μM dexamethasone (Dex)/0.2 μM indomethacin/10 μg/mL insulin and adipogenesis was assessed by Oil Red O staining. Osteogenic differentiation was induced following 21-day culture of cells in MSC medium supplemented with 0.1 μM Dex/0.15 mM ascorbate-2-phosphate/3 mM NaH_2PO_4 and osteogenesis was assessed by alkaline phosphatase (ALP)/von Kossa staining. For chondrogenic induction, MSCs were pelleted in 15-mL tubes and cultured for 21 days in DMEM-High Glucose (Gibco), supplemented with 6.25 μg/mL insulin/6.25 μg/mL transferrin/1.33 μg/mL linoleic acid/1.25 mg/mL bovine serum albumin/1 mM sodium pyruvate/0.17 mM ascorbate-2-phosphate/0.1 μM Dex/0.35 mM L-proline/6.25 ng/mL selenous acid/0.01 μg/mL transforming growth factor-β_3 (R&D Systems) and chondrogenesis was assessed by Alcian blue and Masson's trichrome stains. The reagents for differentiation induction were purchased from Sigma unless otherwise indicated.

Total RNA isolated from differentiated MSCs from patients and controls (RNeasy mini kit; QIAGEN, GmbH, Hilden, Germany) was reverse-transcribed (SUPERSCRIPT II; Gibco) and amplified by polymerase chain reaction (RT-PCR) for the evaluation of specific, differentiation-associated gene expression: adipose fatty acid-binding protein (aP2) and peroxisome proliferator-activated receptor-γ (PPARG) for adipocytes, ALP and runt-related transcription factor 2 (RUNX2) for osteocytes, collagen type II (COL2A1), and aggrecan (AGC1) for chondrocytes. Products were normalized according to β_2-microglobulin (β_2m) expression, using the ImageJ densitometry analysis system. Primer sequences and RT-PCR conditions have been reported previously [32].

Immunophenotypic characteristics of MSCs

Trypsinized MSCs from P1–P6 were immunophenotypically characterized using monoclonal antibodies against CD29 (4B4; Cyto-Stat/Beckman-Coulter, Fullerton, CA), CD44 (J173; Immunotech/Coulter, Marseille, France), CD73 (AD2; Becton Dickinson-Pharmingen, San Diego, CA), CD90 (F15.42; Immunotech/Coulter), CD105 (SN6; Caltag, Burlingame, CA), CD146 (P1H12; Becton Dickinson-Pharmingen), CD45 (IMMU19.2; Immunotech/Coulter), CD14 (RMO52; Immunotech/Coulter), and CD34 (QBend10; Beckman-Coulter). Data were processed in an Epics Elite flow cytometer (Coulter, Miami, FL).

Clonogenic potential of MSCs

Colony-forming unit fibroblast assay. A colony-forming unit fibroblast (CFU-F) assay was used for the evaluation of the clonogenic potential of MSCs through P1–P6 [32]. Trypsinized MSCs from P1 to P6 were seeded at 3 different concentrations (50–150 cells) in 60-mm plates for 14 days. CFU-Fs were quantified using Giemsa staining [30]. The number of CFU-F was estimated per 100 MSCs on the basis of the linear regression analysis obtained from the 3 different initial cell concentrations [31].

Limiting dilution assay. A limiting dilution assay (LDA) was used to indirectly evaluate the frequency of MSCs within BMMCs following 6-week culture of 7 different concentrations of BMMCs (250–10,000 cells/well) in 96-well flat bottom plates as previously described [32,33]. Wells with >50 adherent spindle-shaped cells were considered positive and MSC frequency corresponded to the dilution resulting in 37% negative wells [30,34].

Proliferative potential of MSCs

The proliferative potential of MSCs was evaluated by a methyl triazolyl tetrazolium (MTT)-based assay at P2 and also by estimating the population doubling time through P1–P6 [32]. The formula $2^n = N_x/N_0$ was used for the calculation of the population doublings (n) at each passage based on the number of cells counted in the flask after trypsinization (N_x) and the number of cells initially plated (N_0).

T-cell proliferation assay

PB samples from healthy donors were centrifuged on Histopaque-1077 to obtain the mononuclear cells and the CD3$^+$ cell fraction was fractionated by indirect magnetic labeling (magnetic-activated cell sorting; MACS isolation kit, Miltenyi Biotec GmbH, Germany). In each experiment, purity of CD3$^+$ cells was >96% as estimated by flow cytometry. To evaluate the capacity of MSCs from MDS patients and healthy subjects to suppress T-cell proliferative responses, we stimulated 5×10^4 immunomagnetically sorted normal CD3$^+$ cells with phytohemagglutinin (PHA; 2 μg/mL) or interleukin-2 (IL-2; 500 IU/mL) in the presence or absence of 10^4 irradiated (30 Gy) BM MSCs from MDS patients or allogeneic healthy controls in V-bottomed 96-well culture plates for 7 days in 0.2 mL RPMI-1640 medium (Gibco) containing 10% FCS [35,36]. T-cell proliferation was measured on day 7 following an 18-h pulse with 1 μCi/well ^{3}H-thymidine (^{3}H-TdR; Amersham, Buckingham, UK). ^{3}H-TdR incorporation was measured by using a liquid scintillation counter (LS1701 beta counter-Beckman, USA). The percentage of inhibition of T-cell proliferation by MSCs was calculated by dividing the difference of counts per minute (cpm) between cultures of T cells with and without MSCs in the presence of the activator by the cpm obtained from the cultures of T cells with the activator alone. Experiments were performed in triplicates.

Cytogenetic analysis of BM hematopoietic cells and culture-expanded MSCs

Chromosome-banding analysis. BM cells from MDS patients and healthy controls were cultured in RPMI-1640

medium supplemented with 5% FCS and 100 IU/mL PS at 37°C for 24 and 48 h. For chromosome preparation, colcemide (0.1 g/mL) was added for 2 and 24 h before harvesting. Following treatment with hypotonic KCl solution and fixation with a mixture of glacial acid and absolute methanol (1:3), a modified chromosome-banding technique (GAG; Giemsa bands by acetic saline-Giemsa) was used and 15–25 metaphase cells (range 5–25) were analyzed and classified according to the International System for Human Cytogenetic Nomenclature (ISCN) [37,38]. Similarly, MSCs from P2 were exposed to colcemide (0.1 g/mL) for 24 h and trypsinized. For chromosomal analysis, GAG banding was

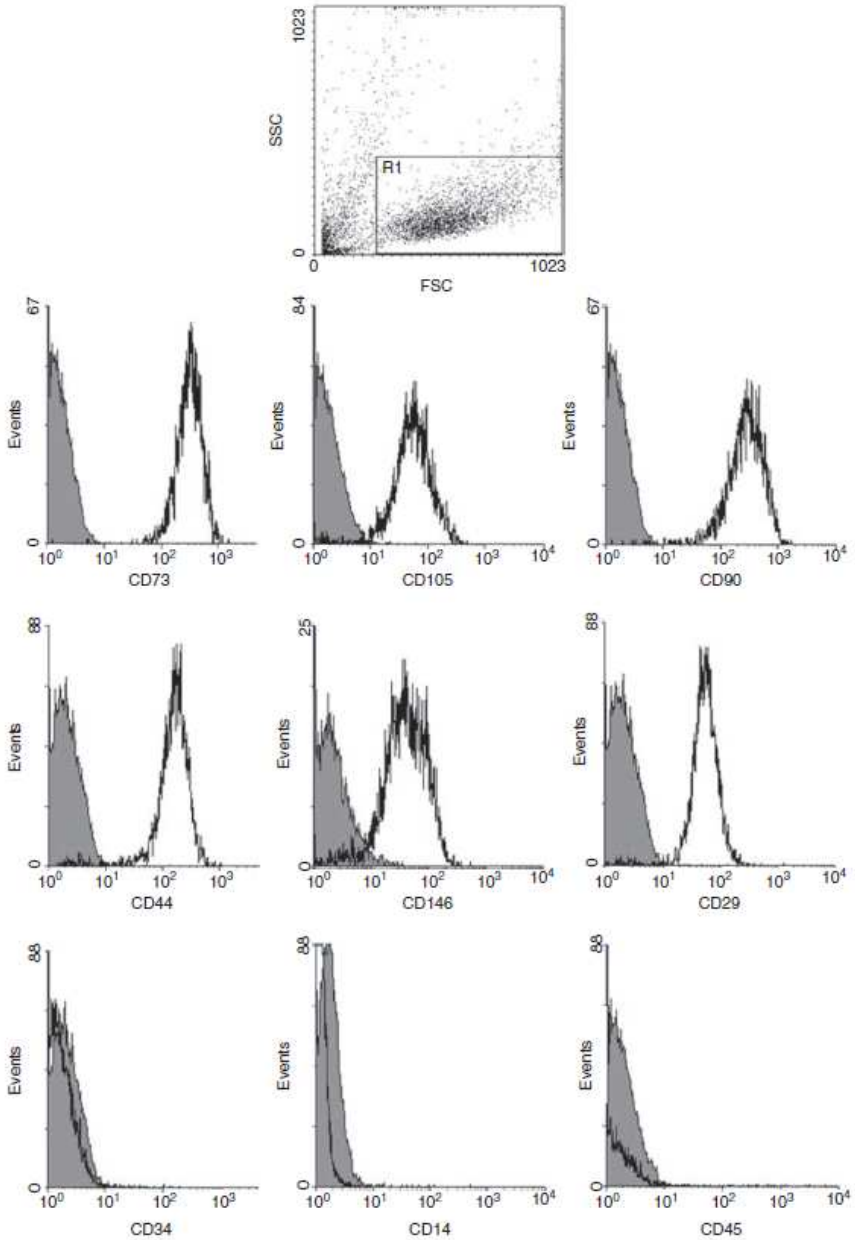

FIG. 1. Immunophenotypic characteristics of mesenchymal stem cells (MSCs). The graphs show the flow cytometric characteristics of MSCs at passage 2 from a representative myelodysplastic syndromes (MDS) patient. The forward scatter (FSC) versus side scatter (SSC) dot plot depicts the gate of MSCs population (R1). The open histograms show the expression of positive (CD73, CD105, CD90, CD44, CD146, CD29) and negative (CD34, CD14, CD45) surface markers in comparison to isotype-matched controls (gray-filled histograms) in MSCs gated in R1.

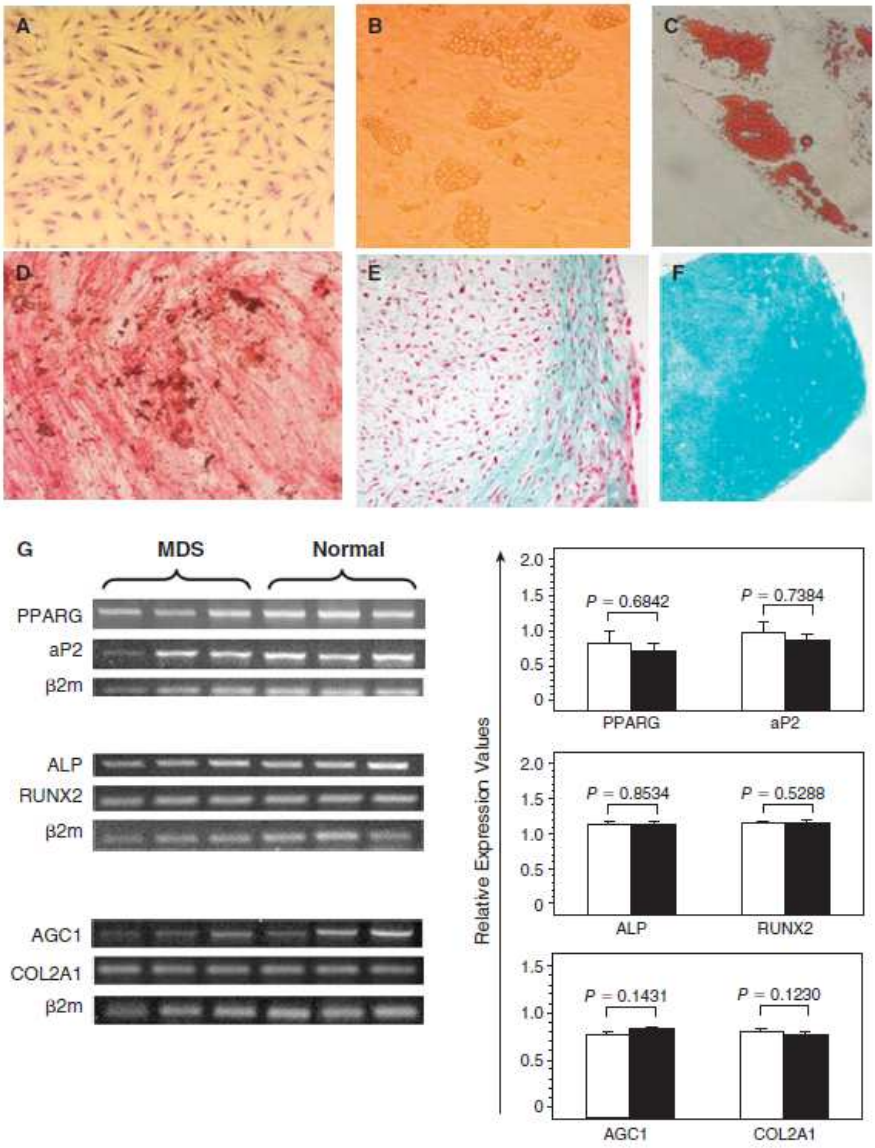

FIG. 2. Differentiation potential of mesenchymal stem cells (MSCs). The upper panel shows undifferentiated MSCs from P2 (**A**) and differentiated cells toward the adipogenic (**B** and **C**), osteogenic (**D**), and chondrogenic (**E** and **F**) lineages from a representative myelodysplastic syndromes (MDS) patient. Adipogenenesis was induced upon culture in medium supplemented with 1-methyl-3-butylisoxanthine/dexamethasone/indomethacin/insulin and was assessed by morphologic evaluation of unstained (**B**) and Oil Red O-stained (**C**) cells. Osteogenesis was induced following culture in medium supplemented with dexamethasone/ascorbate-2-phosphate/NaH_2PO_4 and was assessed by ALP/von Kossa staining (**D**). Chondrogenesis was induced in MSC pellets cultured in the presence of insulin/transferrin/linoleic acid /bovine serum albumin/sodium pyruvate/ascorbate-2-phosphate/dexamethasone/l-proline/selenous acid/transforming growth factor-β_3 and was assessed by Masson (**E**) and Alcian blue (**F**) staining. The graph G in the lower panel depicts specific gene mRNA expression (on the left) of P2 BM MSCs upon differentiation toward the adipogenic (aP2 and PPARG), osteogenic (ALP and RUNX2), and chondrogenic (COL2A1 and AGC1) lineages in representative MDS ($n = 3$) and control ($n = 3$) subjects as well as cumulative data (mean relative values ± SEM) of specific gene mRNA expression from all MDS (■) and controls (□) studied (on the right). Comparison between patients and controls has been performed by means of the nonparametric Mann–Whitney test. Abbreviations: AGC1, aggrecan; ALP, alkaline phosphate; aP2, adipose fatty acid-binding protein; COL2A1, collagen type II; PPARG, peroxisome proliferator-activated receptor-γ; RUNX2, runt-related transcription factor 2.

performed in all 13 MDS patients and in 5 healthy individuals. In 6 MDS patients, a chromosome analysis was also performed in MSCs from P4, P6, and P8. Whenever possible, 15–20 metaphase cells in each passage (range 0–40, median 13) were analyzed and classified according to the ISCN.

A chromosomal aberration was defined as clonal abnormality when at least 2 metaphases were demonstrating the same structural rearrangement or chromosome gain, whereas a chromosome loss had to be identified in at least 3 metaphases [38].

Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) was performed using commercially available locus-specific probes for chromosome 5 [LSI EGR1(5q31)/D5S23,D5S721], chromosome 7 [LSI D7S486(7q31)/CEP7], and chromosome 8 (CEP8) following the protocol of the manufacturer (Abbott Laboratories, Abbott Park, Illinois). The signals of at least 200 interphase nuclei in each case were visualized and counted with a Zeiss Axioskop microscope (Zeiss, Jena, Germany) and documented with the analyzing system ISIS (MetaSystems, Altlussheim, Germany).

Statistical analysis

Statistical analysis was performed by means of the nonparametric Mann–Whitney test and the 2-way analysis of variance (ANOVA) test using the GraphPad Prism statistical software (San Diego, CA). Grouped data were expressed as means ± 1 standard deviation.

Results

Reserves, morphologic, and immunophenotypic characteristics of BM MSCs

MSCs cultures were successfully expanded in all MDS patients and healthy controls. The immunophenotypic analysis at the end of each passage demonstrated that cultures constituted of a homogenous cell population positive for CD73, CD90, CD146, CD105, CD29, CD44 surface antigens and negative for the hematopoietic markers CD45, CD14, and CD34 (Fig. 1). MSCs displayed also the characteristic spindle-like morphology (Fig. 2A). The estimated frequency of MSCs in the BMMC fraction according to the LDA did not differ significantly between MDS patients (21.74 ± 13.59 per 10^5 BMMCs) and healthy controls (24.84 ± 15.43 per 10^5 BMMCs; $P = 0.7823$). These data suggest that the reserves, morphologic, and immunophenotypic characteristics of BM MSCs in MDS patients are similar to the normal individuals.

Differentiation potential of MSCs

Culture-expanded MSCs from MDS patients were able to differentiate into adipogenic, osteogenic, and chondrogenic lineages as was shown by the respective cytochemical

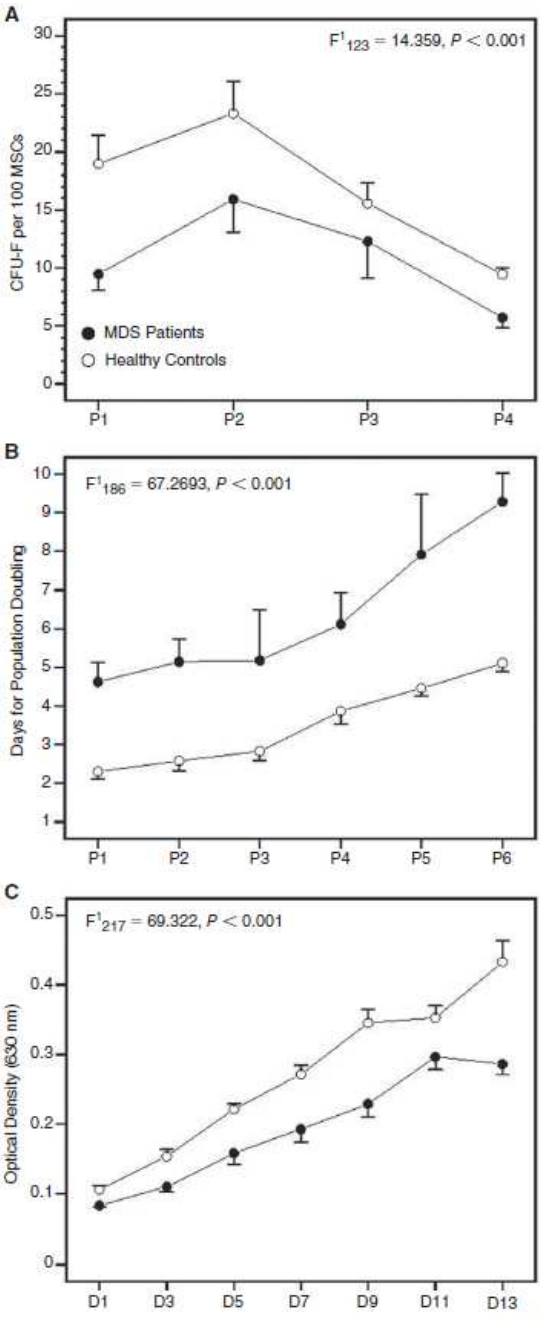

FIG. 3. Clonogenic and proliferative potential of mesenchymal stem cells (MSCs) time course. (**A**) Mean number (±SEM) of CFU-F generated from MSCs over passages in myelodysplastic syndromes (MDS) patients and healthy controls using a 14-day clonogenic assay. (**B**) Days (mean ± SEM) for population doubling through passages as estimated by the cell yield at the end of each passage compared to cells initially plated. (**C**) Results of optical density (mean ± SEM) corresponding to the number of live cells over 13-day culture using the MTT assay in MSCs from passage 2. Comparison between patients and controls was performed using 2-way ANOVA. Abbreviations: D, days in culture; P, passages; SEM, standard error of the mean.

staining (Fig. 2B–2F). Furthermore, the adipogenic, osteogenic, and chondrogenic differentiating capacity assessed by the relative mRNA expression of aP2 and PPARG, ALP and RUNX2, COL2A1 and AGC1, respectively, did not differ significantly between MDS patients and controls ($P = 0.7394$ and $P = 0.6842$, $P = 0.8534$ and $P = 0.5288$, $P = 0.123$ and $P = 0.1431$, respectively; Fig. 2G). These data suggest that BM MSCs from MDS patients display normal differentiation potential.

Clonogenic and proliferative potential of MSCs

Data showing the clonogenic and proliferative potential of MSCs in MDS patients and healthy controls are presented in Figure 3. CFU-F recovery by culture-expanded MSCs was significantly lower in MDS patients compared to controls through P1–P6 culture period ($F = 14.359$, $P < 0.01$) suggesting defective clonogenic capacity of patient MSCs. The doubling time of MSCs during the P1–P6 culture period was significantly increased in MDS patients compared to controls ($F = 67.58405$, $P < 0.001$). Specifically, the MSC doubling time ranged from 4.62 ± 1.85 days (P1) to 9.28 ± 2.71 days (P6) in patients and from 2.31 ± 0.87 days (P1) to 5.06 ± 1.03 days (P6) in controls. In accordance with the cell doubling time data were the results from MTT assay at a representative passage (P2). The number of live cells corresponding to the obtained optical density gradually increased over the 13-day culture period, however, remained significantly lower in MDS patients compared to controls ($F = 69.3220$, $P < 0.001$) corroborating further the defective proliferative potential of MSCs in MDS.

Immunosuppressive properties of MSCs

To determine whether patient MSCs could efficiently inhibit T-cell proliferative responses, we mixed normal or patient MSCs with allogeneic normal T lymphocytes in the presence of PHA or IL-2 and we measured ^{3}H-TdR incorporation. As shown in Figure 4, a significant reduction was obtained in T-cell proliferation when cultures of purified T lymphocytes stimulated by PHA or IL-2 (3,377 ± 1,674 cpm and 16,934 ± 24,777 cpm, respectively) were performed in the presence of allogeneic normal MSCs (302 ± 99 cpm and 1,753 ± 1,734 cpm, respectively; $P = 0.0034$ and $P = 0.0317$, respectively). Similarly, T-cell proliferative responses to the above activators (21,608 ± 22,610 cpm and 56,211 ± 41,093 cpm, respectively) were significantly reduced in the presence of MSCs from MDS patients (657 ± 281 cpm and 10,561 ± 13,733 cpm, respectively; $P = 0.0079$ and $P = 0.0463$, respectively). The percentage of inhibition of PHA-induced or IL-2-induced T-cell proliferation by MSCs did not differ significantly between patients (90.59% ± 8.04% and 84.14% ± 10.68%) and controls (88.53% ± 6.45% and 82.39% ± 12.25%, respectively; $P = 0.6905$ and $P = 1.0$, respectively). Taken together these data suggest that MSCs from MDS patients display normal immunosuppressive properties in terms of the capacity to inhibit T-cell proliferation induced by mitogens.

Cytokine production by MSCs

To investigate whether BM MSCs are the primary source of the proinflammatory cytokines known to be associated with the pathophysiology of MDS, we assessed TNF-α, IL-1β, and IL-6 levels in culture supernatants from P2, P4, and P6 (Table 2). No statistically significant difference was found between patients and controls in the levels of cytokines time course. Furthermore, the levels of the growth-promoting cytokines VEGF and SDF-1α during the P2–P6 culture period did not differ significantly between patients and controls (Table 2) suggesting that abnormal cytokine production by MSCs per se seems unlikely to be the cause of the impaired clonogenic and replicative capacity of MSCs in MDS.

Cytogenetic analysis of BM hematopoietic cells and MSCs

Cytogenetic analysis in MDS patients. Results from the cytogenetic analysis of patient BM hematopoietic cells and

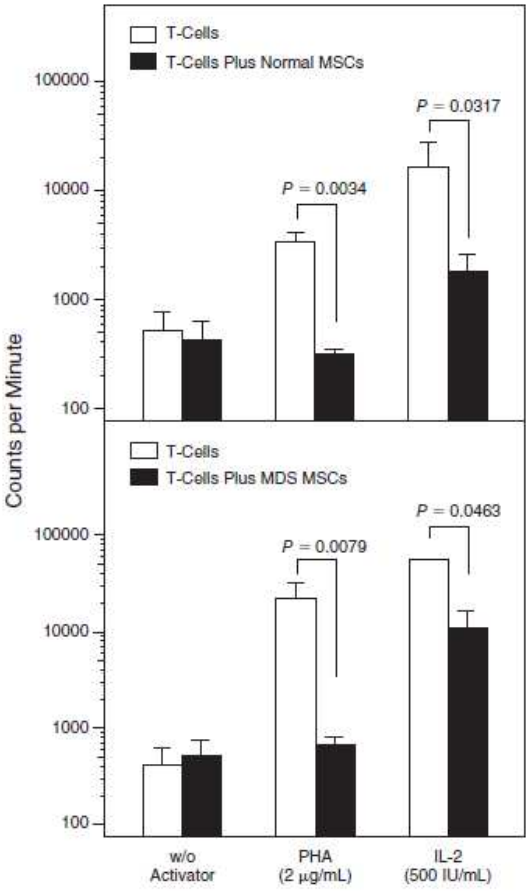

FIG. 4. Inhibitory effect of mesenchymal stem cells (MSCs) on T-lymphocyte proliferation. The bars represent the proliferative responses (expressed in counts per minute upon ^{3}H-TdR incorporation) of unstimulated and activator-induced T lymphocytes in the presence or absence of BM MSCs from allogeneic normal (upper panel) or patient (lower panel) MSCs. Data are expressed as mean (±SEM) of triplicates of 5 separate experiments. Comparison of counts per minute in the presence or absence of MSCs was performed by means of the nonparametric Mann–Whitney test and the P values indicating the statistical significance are shown.

Table 2. Cytokine Levels in the Supernatants of MSC Cultures Time Course

	MDS patients (n = 13)			Healthy controls (n = 20)			
	P2	P4	P6	P2	P4	P6	P value[a]
TNF-α (pg/mL)	0.24 ± 0.03	0.23 ± 0.02	0.23 ± 0.05	0.22 ± 0.08	0.28 ± 0.20	0.23 ± 0.03	N.S.
IL-1β (pg/mL)	0.95 ± 3.22	0.62 ± 1.19	0.92 ± 1.06	2.54 ± 2.30	0.23 ± 0.34	0.78 ± 0.69	N.S.
IL-6 (ng/mL)	6,521 ± 3,604	6,481 ± 2,863	6,189 ± 3,636	5,061 ± 2,116	7,514 ± 3,295	5,443 ± 3,349	N.S.
VEGF (pg/mL)	2,401 ± 872	1,683 ± 761	2,063 ± 798	2,035 ± 791	1,711 ± 1,095	1,711 ± 602	N.S.
SDF-1α (pg/mL)	3,435 ± 2,312	3,375 ± 1,711	2,959 ± 1,097	3,842 ± 1,642	3,193 ± 1,339	2,476 ± 1,133	N.S.

Measurements have been performed by means of an enzyme-linked immunosorbent assay.
Cytokine levels between MDS patients and healthy controls time course were performed by 2-way ANOVA.
Abbreviations: IL, Interleukin; MDS, myelodysplastic syndromes; MSCs, mesenchymal stem cells; N.S. non-significant difference; P, passage; SDF-1α, stromal-derived factor-1α; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

MSCs are shown in Table 1. In total, karyotype abnormalities were identified in BM hematopoietic cells in 8 out of 13 MDS patients. Specifically, 5 patients displayed interstitial deletion of the long arm of chromosome 5 [del(5q)] with an additional del(7q) subclone in 1 patient, 2 patients had trisomy 8 (+8), and 1 patient −Y. The chromosomal analysis of the corresponding MSCs did not show any of these abnormalities at P2–P8. The presence of del(5q) and trisomy 8 in hematopoietic cells and their absence in MSCs was verified by FISH analysis (data not shown). Interestingly, cultures of patient MSCs showed a clonal trisomy 5 in 3 cases and trisomy 7 in one case. Although the trisomy 5 was identified in only 1 out of 4 passages (P4 in one case and P6 in 2 cases) with classical cytogenetics, FISH analysis revealed the existence of the trisomy 5 in all passages analyzed (P2, P4, P6, P8; Table 3). Trisomy 7 was identified only in the only passage tested (P2) and was confirmed by FISH analysis. The absence of trisomies 5 and 7 in the corresponding hematopoietic cell cultures was confirmed by FISH analysis (data not shown).

Cytogenetic analysis of healthy subjects. As anticipated, chromosome analysis of BM hematopoietic cells from healthy individuals showed normal karyotype (Table 4). The chromosome analysis of the respective MSCs, however, showed a clonal trisomy 5 in one case at P2 (Fig. 5). Pre-existence of trisomy 5 in BM hematopoietic cells of this subject was excluded by FISH.

Because trisomies 5 and 7 were only identified in a small number of analyzed metaphases and in only 1 out of 4 passages in 4 patient and one control MSC cultures, while a FISH screening revealed the existence of trisomy 5 or 7 in interphase cells of all passages, an additional FISH screening with probes for chromosomes 5 and 7 was performed in all the remaining cases with normal MSC karyotype (healthy and patients MSCs) in all passages. No further cases with a masked trisomy 5 or 7 were identified. Taken together, these data show that although BM MSCs from MDS patients do not show the cytogenetic abnormalities present in BM hematopoietic cells, they may show irrelevant clonal abnormalities during passages. The pathophysiologic significance of this abnormality, however, is questionable because a similar abnormality was also identified in normal MSCs.

Table 3. FISH Screening Analysis From MSC Cultures Showing Karyotypic Abnormalities

No	FISH marker	MSC passage	% of aberrant BM MSCs
Pt-1	+5	2	46%
		4	47%
		6	21%
		8	2%
Pt-2	+5	2	70%
		4	70%
		6	62%
		8	60%
Pt-3	+5	2	10%
		4	6%
		6	65%
		8	4%
Pt-10	+7	2	26%
ND-1	+5	2	38%

Abbreviations: BM, Bone marrow; FISH, fluorescence in situ hybridization; MSCs, mesenchymal stem cells; ND, normal donor; No, number; Pt, patient.

Discussion

The mechanisms leading to hematopoietic failure in patients with MDS are not entirely known; however, there is evidence suggesting that not only intrinsic abnormalities within the HSC compartment but also defective stromal support mechanisms may have a role [39]. In this study, we have evaluated the quantitative, functional, and cytogenetic characteristics of BM MSCs in patients with de novo MDS aiming to explore whether the BM stromal cells are primarily affected in these patients. The existing data on this field of research are limited and contradictory.

MSCs from all MDS patients studied were expanded successfully and were morphologically and immunophenotypically indistinguishable from the respective of the healthy controls. These observations are in agreement with data of Flores-Figueroa et al. also showing normal development and immunophenotypic characteristics of MDS-derived MSCs [21] whereas are in contrast with data of Lopez-Villar et al. suggesting that MDS MSCs occasionally fail to grow in vitro [26]. These inconsistencies might be attributed to the heterogeneity of MDS patients used in these studies in terms of WHO type and IPSS risk category. Despite the successful expansion of patient MSCs, we found that the rate of cellular growth through passages, assessed by the population doubling time, was significantly reduced in MDS patients compared to controls, suggesting defective proliferative capacity of MSCs. This finding was further substantiated by the MTT

TABLE 4. CYTOGENETIC DATA OF NORMAL BM HEMATOPOIETIC CELLS AND MSCS

No	Age	Karyotype BM Hematopoietic Cells	MSC passage	Karyotype BM MSCs
ND-1	80	46,XY [20]	2	47,XY,+5 [5]/46,XY [29]
ND-2	62	46,XY [25]	2	46,XY [5]
ND-3	75	46,XY [23]	2	46,XY [20]
ND-4	70	46,XY [20]	2	46,XY [15]
ND-5	83	46,XY [20]	2	46,XY [5]

Abbreviations: BM, Bone marrow; MSCs, mesenchymal stem cells; ND, normal donor; No, number.

assay that also showed low rate of cell proliferation of MDS-derived MSCs in comparison to normal subjects as well as by the CFU-F clonogenic assay that revealed defective colony formation of patient MSCs through passages compared to normal MSCs. These abnormalities might represent a primary MSC defect or a premature cellular replicative exhaustion secondary to the long-term inflammatory process present in patients' BM. Similar abnormalities have been described in BM MSCs from patients with rheumatoid arthritis and have been attributed to shortened telomere length and cellular senescence associated with the inflammatory BM microenvironment [32].

Using a LDA, we found that the frequency of BM MSCs within the BMMC fraction was similar in patients and healthy controls suggesting normal reserves of MSCs in MDS BM. Regarding the MSC differentiation capacity, we showed that culture-expanded MSCs from the patients displayed normal osteogenic, chondrogenic, and adipogenic potential. Previous studies have suggested impaired chondrogenic or adipogenic potential of BM MSCs in MDS based mainly on the morphologic evaluation of immunohistochemically

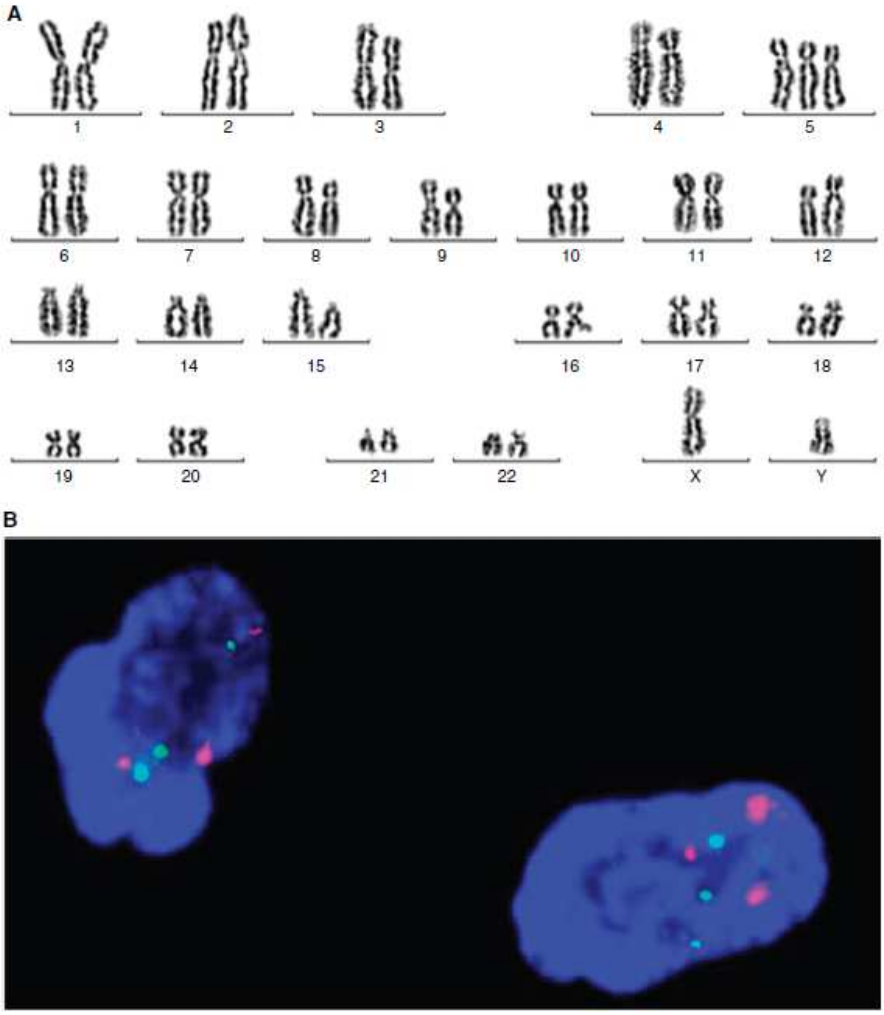

FIG. 5. Cytogenetic analysis of mesenchymal stem cells (MSCs) derived from the normal donor-1 at P2. (**A**) Chromosome-banding analysis showing trisomy 5. (**B**) FISH analysis with a dual color probe LSI EGR1 (Spectrum Orange 5q31)/D5S23,D5S721 (SpectrumGreen, 5p15.2) showing 3 signals on 2 interphase nuclei from MSCs.

stained differentiated cells [25,26]. However, in our study we have evaluated the differentiation potential of MSCs based not only on the assessment of the morphological and cytochemical characteristics of cells upon differentiation but also on the quantification of differentiation-related specific gene mRNA expression. Normal differentiation potential of MDS-derived BM MSCs has been also proposed by others based on cytochemical findings [21].

MSCs normally display immunosuppressive properties and inhibit T-cell proliferation to alloantigens and mitogens [40]. Since aberrant immune responses and T-cell-mediated inhibition of hematopoiesis have been associated with the pathophysiology of MDS [10], we evaluated the capacity of BM MSCs from MDS patients to suppress T-cell immune responses in vitro. In contrast to previous reported data suggesting impaired immunosuppressive potential of MDS-derived BM MSCs [24,41], we found that patient MSCs sufficiently inhibited T-cell proliferation induced by mitogens. The discrepancy might be attributed to differences in the methodology. Our data suggest that BM MSCs is unlikely to have a major role in the aberrant T-cell responses occasionally seen in MDS patients. In accordance with this assumption was the normal production of TNF-α, IL-1β, and IL-6 by MSC stromal layers time course suggesting that the BM MSCs per se are not the main cellular source of these proinflammatory cytokines in patients' BM. Similarly, the levels of the growth-promoting cytokines VEGF and SDF-1α in patient MSC culture supernatants were within normal range time course.

The chromosome-banding analysis of BM hematopoietic cells revealed clonal abnormalities in 8 out of 13 MDS patients and, as expected, normal karyotype in 5 healthy controls studied. Chromosomal analysis of the corresponding MSC cultures showed karyotype abnormalities in 4 out of 13 MDS patients (trisomies 5 and 7) and in 1 out of 5 healthy individuals (trisomy 5) but different from those found in the BM hematopoietic cells. These data indicate that MSC cultures from MDS patients do not show the cytogenetic abnormalities present in the BM hematopoietic cells at diagnosis and, accordingly, they do not belong to the abnormal clone. Available data on the cytogenetic analysis of MSCs from MDS patients are controversial probably due to the methodology diversities. There are studies suggesting that MDS-derived MSCs do not harbor the cytogenetic aberrations present in the corresponding BM hematopoietic cells [20,42] and other showing clonal cytogenetic abnormalities [22,23,26]. Furthermore, different groups have shown a big variety in the kind of aberration reported, that is hypodiploidy, balanced translocations, whole chromosome gains, and deletions. Notably, most of the reported aberrations in BM MSCs are non-characteristic for MDS [43].

We have shown that patient MSC cultures displayed chromosomal stability until late passages. This finding is in accordance with results from other groups reporting normal karyotype until late MSC passages from patients with different hematologic disorders including MDS and healthy individuals [44,45]. The trisomy 5, found in MSC cultures from 3 MDS patients and one healthy control, has been described in acute lymphoblastic and myeloid leukemias as well as in neoplastic and non-neoplastic solid tumors [46–50]. Trisomy 5 has also been reported as a probable nonrandom phenomenon in long-term cultures from solid tumor patients [51]. Because trisomy 5 was identified in both patient and normal MSC cultures and chromosome gains have already been described in cell cultures of normal tissues [52,53], we assume that the abnormality probably represents a nonrandom in vitro phenomenon with presently unknown significance. Trisomy 7, found in MSC cultures from one MDS patient, has not been described in hematologic malignancies [54,55] but it is a common finding in malignant and non-malignant tissues [56–58]. It has been suggested, however, that trisomy 7 accumulates with age and may be associated with age progression rather than with a disease state [56]. Accordingly, the pathophysiologic significance of this abnormality in patient's MSC cultures remains uncertain.

In conclusion, data from this study show that BM MSCs from patients with de novo MDS are normal in regards to the number, the differentiation potential, and immunosuppressive properties. Patient MSCs do not show any aberrations in the production of proinflammatory or growth-promoting cytokines and do not harbor the cytogenetic abnormalities present in hematopoietic cells. Patient MSC cultures display chromosomal stability time course, however, they may develop irrelevant chromosomal alterations with unknown pathophysiologic significance as they may also occur in MSC cultures from normal individuals. Finally, MDS-derived MSCs display impaired proliferative and clonogenic potential probably associated with the chronic inflammatory process present in patients' BM. Overall, our data suggest that BM MSCs from MDS patients do not belong to the abnormal clone and do not display major quantitative and functional abnormalities. The underlying mechanism for the impaired proliferative/clonogenic potential and the pathophysiologic significance of the subtle chromosomal alteration occurred during culture is an interesting field for further investigation.

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Author Disclosure Statement

The authors declare no conflict of interest.

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