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Multipotent adult progenitor cells improve the hematopoietic function in myelodysplasia

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Abstract

Background aims—Myelodysplastic syndromes (MDS) are a group of clonal stem cell disorders affecting the normal hematopoietic differentiation process and leading to abnormal maturation and differentiation of all blood cell lineages. Treatment options are limited, and there is an unmet medical need for effective therapies for patients with severe cytopenias.

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Methods—We demonstrate that multipotent adult progenitor cells (MAPC) improve the function of hematopoietic progenitors derived from human MDS bone marrow (BM) by significantly increasing the frequency of primitive progenitors as well as the number of myeloid colonies.

Results—This effect was more pronounced in a non-contact culture, indicating the importance of soluble factors produced by the MAPC cells. Moreover, the cells did not stimulate the growth of the abnormal MDS clone, as shown by fluorescent *in situ* hybridization analysis on BM cells from patients with a known genetic abnormality. We also demonstrate that MAPC cells can provide stromal support for patient-derived hematopoietic cells. When MAPC cells were intravenously injected into a mouse model of MDS, they migrated to the site of injury and increased the hematopoietic function in diseased mice.

Discussion—The preclinical studies undertaken here indicate an initial proof of concept for the use of MAPC cell therapy in patients with MDS-related severe and symptomatic cytopenias and should pave the way for further investigation in clinical trials.

Keywords

cell therapy; multipotent adult progenitor cells; myelodysplasia

Introduction

Myelodysplastic syndromes (MDS) are heterogeneous clonal hematopoietic stem cell (HSC) disorders characterized by ineffective hematopoiesis and an increased probability of developing acute myeloid leukemia [1]. This group of disorders is the most common hematological stem cell disorder and the age-adjusted annual MDS incidence varies between 3–4 per 100 000 per year, but increases to 35 per 100 000 per year in patients 70 years or older [2]. Symptoms arise from anemia, neutropenia and thrombocytopenia. The etiology of bone marrow (BM) failure in MDS is not yet fully understood but is multifactorial and caused by intrinsic defects in the HSC (e.g., mutations in *AML1*, *EVII*, *NUP98* and *NUP214*) as well as extrinsic defects in the BM niche [3]. The latter include excess production of, for example, tumor necrosis factor- α and interferon- γ , abnormalities in endothelial cells, mesenchymal stromal cells (MSCs) and osteoblasts (cells that are part of the HSC niche) and activation of T lymphocytes, monocytes and dendritic cells leading to abnormal proliferation and differentiation of HSCs [4–9]. The only curative therapy is allogeneic HSC transplantation, which is suitable for only a minority of the patients due to advanced age. Recently, three drugs have been approved by the U.S. Food and Drug Administration: decitabine, lenalidomide and 5-azacitidine (the last two are also approved by the European Medicines Agency); in a subgroup of MDS patients, these drugs decrease cytopenias, induce cytogenetic remission and reduce BM blasts, while not completely eradicating the malignant HSC clone [10]. Despite these treatment options there is still a large, unmet medical need for effective therapies for patients with severe MDS-related cytopenias.

Multipotent adult progenitor cells (MAPC) are adult BM-derived stromal stem cells. Compared with MSCs, they have significantly greater proliferation potential and differentiation potential (e.g., functional endothelium *in vitro* as well as *in vivo*) [11]. Like

MSCs, MAPC cells have potent immunomodulatory effects in T cells [12–14], are non-immunogenic for T-cell proliferation and cytokine production and even suppress T-cell activation and proliferation induced by alloantigens and mitogens *in vitro*. Human MAPC cells also have inhibitory effects in natural killer cells [15].

Because of the potent immunomodulatory and anti-inflammatory effects of MAPC cells and their capacity to enhance hematopoietic recovery following transplantation [16], we hypothesized that MAPC cells could be used as a novel therapy in MDS patients to decrease cytopenias because they might secrete factors that enhance hematopoiesis, reduce inflammation and/or replace defective niche cells.

Here, the effect of MAPC cells on the function of hematopoietic progenitor/stem cell (HSPC)-derived MDS BM was assessed *in vitro*. We also transplanted MAPC cells in a mouse model of MDS, demonstrating homing to BM and improvement of hematopoiesis *in vivo*.

Methods

Cell culture

Briefly, MAPC cells were maintained under hypoxic conditions (37°C, 5.5% CO₂ and 5% O₂) at a density of 2 000 cells/cm² and were split every 2–3 days. The medium consisted of Dulbecco's Modified Eagle's Medium low glucose (Gibco, Invitrogen), MCDB-201 (Sigma-Aldrich) supplemented with dexamethasone, L-ascorbic acid, insulin-transferrin-selenium, linoleic acid-bovine serum albumin (all from Sigma-Aldrich), penicillin/streptomycin (Gibco, Invitrogen), along with serum supreme (Lonza BioWhittaker) and human platelet-derived growth factor-BB (R&D Systems) and epidermal growth factor (Sigma-Aldrich).

The stromal cell lines AFT024 and UG26-1B6, kind gifts from Dr. K. Moore (Mount Sinai, New York) and Dr. E. Dzierzak (Erasmus MC, Rotterdam), respectively, were grown on 0.1% gelatin-coated plates in Myelocult medium (Stem Cell Technologies) supplemented with 15% fetal calf serum (Sigma Aldrich), 35% α MEM + Glutamax1, 1% penicillin/streptomycin and 20 mmol/L β -mercaptoethanol (all from Gibco, Invitrogen). Cultures were kept at 33°C and 5.5% CO₂.

Human hematopoietic cell assays

Human BM was harvested from the iliac crest of patients and healthy donors after obtaining informed consent in accordance with the Medical Ethics Committee of the University Hospitals Leuven (UZ Leuven), which comply with the Helsinki declaration.

Isolation of BM mononuclear cells (BM-MNCs) and CD34⁺ cells was done using standard procedures. Briefly, BM-MNCs were separated by Ficoll-Paque (GE Healthcare) density gradient centrifugation (specific gravity 1.077 g/mL) and CD34⁺ cells selected by magnetic activated cell sorting technology (Miltenyi Biotec) according to the manufacturer's instructions. Purity ranged from 85 to 95%.

Human colony forming cell (CFC) assays were performed by mixing BM-MNCs with methylcellulose containing recombinant granulocyte-macrophage colony-stimulating factor, interleukin (IL)-3, stem cell factor (SCF) and erythropoietin (EPO) (Methocult H4434, Stem Cell Technologies). The total number of cells seeded was 15 000 BM-MNCs per well for healthy donors and 150 000 BM-MNCs/well for patients in a 12-well plate (Corning), all in triplicate. Cultures were incubated at 37°C, 5.5% CO₂ and scored between day 14 and 16 by inverted microscopic examination. Colonies were classified as granulocyte/macrophage (CFU-GM), erythroid (bursting form unit-erythroid [BFU-E]) or multipotent (CFU-granulocyte-erythrocyte-monocyte-megakaryocyte [GEMM]) progenitor cells.

For long-term culture-initiating cell (LTC-IC) assays, 15 000 CD34⁺ cells per well were plated on confluent, irradiated (25 Gy) AFT024 feeders in LTC-IC medium (Dulbecco's Modified Eagle's Medium high glucose [Gibco, Invitrogen] supplemented with 20% horse serum, 1% penicillin/streptomycin, 20 mmol/L β-mercaptoethanol [all from Gibco, Invitrogen] and 10⁻⁵ mol/L hydrocortisone [Stem Cell Technologies]) in a 12-well plate, which was incubated at 33°C, 5.5% CO₂. Each week, half of the medium was removed and replaced with fresh medium and hydrocortisone. After 5 weeks, adherent and nonadherent cells were harvested and assayed in the CFC assay as described earlier. On the basis of the number of colonies the total number of LTC-ICs per 15 000 plated CD34⁺ progenitor cells was calculated according to the manufacturer's instructions.

In some conditions, 10 000 irradiated (25 Gy) MAPC/cm² were plated in 0.4-μm transwell inserts above CD34⁺ cells (non-contact), or 5 000 irradiated (25 Gy) MAPC/cm² were plated in contact with CD34⁺ cells. Half medium changes were done every week. In experiments where MAPC cells were used as stromal feeders, they were grown in expansion medium until they reached confluency and then irradiated (25 Gy).

MDS mouse model

C57Bl/6-CD45.1 and -CD45.2 mice were purchased from Charles River Jackson Laboratories. All mice were kept and/or bred in the animal facility of the KU Leuven and approved by the ethical committee for animal experiments at the KU Leuven.

Lin⁻ cells were isolated from 8- to 13-week-old CD45.1 mice with a Lin⁻ cell isolation kit (Miltenyi Biotec) and transduced with supernatants containing a lentiviral vector (LV) encoding *EVII* (pCHMWS-eGFP-IRES-EVI1) or a control vector (pCHMWS). Transduced BM cells were transplanted into 8- to 13-week-old, lethally irradiated (8.5 Gy) CD45.2 mice. Control mice were transplanted with untransduced Lin⁻ cells. Secondary transplantations were carried out in CD45.2 recipient mice using BM cells collected from primary transplanted animals upon appearance of cytopenias. These cells were given in a 3:1 or 5:1 ratio together with wild-type Lin⁻ cells. Overexpression of *EVII* was evaluated monthly by polymerase chain reaction (PCR) on blood samples with primers listed in Table I.

Fluorescent *in situ* hybridization

The mixed MDS BM-derived progeny from four patients with an abnormal karyotype was collected from LTC-IC assays after 5 weeks of culture, grown ± MAPC (non-contact).

Spreads were made from 20 000 cells (in triplicate) using aCytospin 4 Cytocentrifuge (Thermo Scientific), fixed with Carnoy solution and dehydrated. Spreads were denatured at 75°C for 6 min and hybridized overnight at 37°C with probes for 5q, 20q and t(1;3) abnormalities (Abbott Molecular). Spreads were counterstained with 4',6-diamidino-2-phenylindole and washed with Igelpal solution, and the percentage of cells with a mutant karyotype were enumerated.

Blood values and histology of transplanted mice

Peripheral blood (PB) counts of recipient mice were recorded every 2 weeks until the animal was sacrificed or died. Blood was collected (20 µL) in EDTA-coated tubes through tail vein bleeding, and blood counts were measured on a SCIL Vet Abc hematology analyzer (Scil Animal Care Company). Blood and BM smears from recipient mice were made on glass slides and air dried, after which a May-Grünwald-Giemsa staining was performed.

Mouse hematopoietic cell assays

Mouse CFC assay was performed by mixing BM-MNCs with Methocult M3434 (Stem Cell Technologies) supplemented with recombinant SCF, IL-3, IL-6 and EPO. A total number of 2 000 cells per well were seeded in a 12-well plate (Corning), all in triplicate, maintained at 37°C and 5.5% CO₂ and scored between day 10 and 12 by inverted microscopic examination. Colonies were classified as described above.

The mouse LTC-IC assay was performed by plating Lin⁻ cells in serial dilutions (100, 300, 900, 2 700, 8 100, 24 300 cells per well, 10 wells per dilution) in a 96-well plate on confluent, irradiated (25 Gy) UG26-1B6 feeders in LTC-IC medium, incubated at 33°C, 5.5% CO₂. Half medium changes occurred every week. After 5 weeks, medium was removed, and methylcellulose and cytokines were applied to all wells as described earlier. Presence or absence of colonies in each well was scored 14 days later and LTC-IC frequency was calculated by Poisson statistics (L-Calcul statistical software, Stem Cell Technologies).

In vivo bioluminescence imaging

MAPC cells were transduced with a LV encoding triple flag tagged firefly luciferase (Fluc) and the human sodium iodide symporter (hNIS) further referred to as EF1α-Fluc-hNIS [17]. For selection, 400 ng/mL puromycin (Merck Millipore) was added to the growth medium, and the cells were maintained under these conditions.

Transduced MAPC cells ($0.7-1 \times 10^6$, resuspended in 200 µL phosphate-buffered saline) were infused in the tail vein of EVII and control mice. On the day of analysis, animals were anesthetized with 2% isoflurane in 100% oxygen, and D-luciferin was injected subcutaneously (126 mg/kg body weight). Images were acquired using an IVIS Spectrum system (Perkin Elmer). The data are reported as total photon flux (p/s) from a circular region of interest.

Histological analysis of mice transplanted with hNIS transduced human MAPC cells

Animals were sacrificed and femurs isolated for immunohistochemistry and preserved in 4% paraformaldehyde. Samples were decalcified for 12 h using Richard-Allan scientific

decalcifying solution (Thermo Scientific) and embedded in paraffin for sectioning. Before immunohistochemical staining antigens were retrieved (sections were microwaved in a 10-mmol/L citrate buffer at pH 6.0), and endogenous peroxidase activity was quenched with peroxidase blocking buffer (Dako). Samples were incubated with 10% normal goat serum to prevent nonspecific binding. Sections were incubated with an anti-hNIS primary antibody (1:50, Abcam). Secondary goat anti-rabbit horseradish peroxidase-conjugated antibody was used (Dako, EnVision + System, HRP) and immunoreactivity was visualized with 3,3'-diaminobenzidine. Cells were counterstained with Mayer hematoxylin, and stainings were analyzed using a Nikon Eclipse 80i microscope (Nikon).

Human DNA quantification

Genomic DNA was extracted from 6×10^6 murine total BM cells using the Innuprep DNA Mini Kit (Analytik Jena) according to the manufacturer's protocol. The amount of human specific DNA was quantified. Primers are listed in Table 1 and amplify a 141-bp fragment of the human chromosome 17 using platinum Sybrgreen qPCR supermix UDG (Invitrogen) and a real-time PCR system (Applied Biosystems). The amount of human DNA was calculated according to a standard curve using 50%, 5%, 0.5%, 0.05%, 0.005% and 0% human DNA in mouse DNA (final concentration of 100 ng/ μ L).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 5.04 (GraphPad Software Inc.). All data sets were tested for normality using the Kolmogorov-Smirnoff test. For comparison between two groups an unpaired *t*-test or Mann-Whitney *U* test was used. Comparison between more than two groups was calculated by one-way analysis of variance with Bonferroni correction or Kruskal-Wallis with Dunn's multiple comparison test. Survival curves were calculated with the log-rank test. All data are presented as mean $\pm$ SEM, and a *P* value < 0.05 was considered statistically significant.

Results

BM from MDS patients contains significantly fewer CFCs and LTC-ICs compared with BM from age-matched, healthy control subjects

BM was obtained from 20 patients with either confirmed MDS or patients with cytopenias without definite MDS diagnosis and eight healthy, age-matched controls. Thirteen patients belonged to the low-risk and two patients to the high-risk subcategory of MDS according to the World Health Organization classification, and five patients were classified as cytopenia of unknown origin (Table II). BM cellularity was similar in patients (age 72.9 ± 1.9 years) and age-matched, healthy controls (age 55.6 ± 1.5 years), whereas it was significantly higher in very young donors (age 21.2 ± 0.5 years). On the other hand, the percentage of CD34⁺ cells did not differ between any of the groups (Figure 1A). Consistent with previous studies [18], a clear and significant decrease in the number of BFU-E and CFU-GM, and in LTC-IC frequencies was observed in MDS patients compared with healthy donors ($P < 0.0001$ for both assays) (Figure 1B).

MAPC cells increase CFU-GM colony formation and the frequency of LTC-IC in MDS-derived BM

To assess the effect of MAPC cells on the hematopoietic capacity of BM-MNCs derived from MDS patients, MAPC cells were added in a transwell insert above BM-MNCs in the CFC assay. After 14 days, no significant increase in the number of BFU-E could be observed when MAPC cells were added to patient BM-MNC cultures (Figure 1C, upper panel). However, addition of MAPC cells significantly increased the CFU-GM frequency in BM-MNCs of patients by 1.5-fold ($P = 0.047$) (Figure 1C,E, upper panel), which was still 4.5-fold lower than the numbers achieved in healthy, age-matched individuals. Addition of MAPC cells to BM-MNC cultures of healthy donors did not have any impact on colony formation (Figure 1C, lower panel).

Next, to assess the effect of MAPC cells on primitive hematopoietic progenitor cells, CD34⁺ cells of patients and controls were cultured on AFT024 feeders in a LTC-IC assay with or without MAPC cells plated in a transwell insert above the culture. In patients with MDS or cytopenias of unknown origin, but not in control subjects, a significant twofold higher frequency of LTC-ICs was observed when MAPC cells were added ($P < 0.0001$) (Figure 1D,E, lower panel), reaching 50% of the frequency seen in healthy donors.

The positive effect on LTC-IC frequency was MAPC donor-independent, as similar results were obtained in MDS patients with a second MAPC donor ($P = 0.0005$) (Figure 2A). We also found that providing the cells in a transwell insert intermittently (every other week) to the culture or in direct contact could also significantly increase the LTC-IC frequency ($P = 0.0006$ and $P = 0.0091$, respectively), albeit to lower levels than when MAPC cells were continuously provided in a transwell insert (Figure 2A). Finally, providing expansion medium alone (containing serum and growth factors) in a transwell insert did not affect LTC-IC frequency (Figure 2A).

Finally, to assess whether MAPC cells themselves could support hematopoiesis, CD34⁺ cells from patients were grown on MAPC feeders in an LTC-IC assay. MAPC feeders supported LTC-IC generation from CD34⁺ cells, but numbers were generally three times lower than when AFT024 feeders were used ($P < 0.0001$) (Figure 2B).

MAPC cells do not favor the proliferation of cytogenetically abnormal clones

We assessed whether MAPC cells caused a preferential clonal differentiation of the abnormal MDS clone that could potentially enhance leukemic evolution. We cultured CD34⁺ cells from BM of four patients with a known cytogenetic abnormality in an LTC-IC assay with or without MAPC cells in a transwell insert above the culture. After 5 weeks, BM-derived progeny was collected for Fluorescent *in situ* hybridization (FISH) analysis and the percentage of cytogenetically abnormal cells was enumerated in cultures. A decreased or similar percentage of abnormal cells was detected when MAPC cells were added to the culture, indicating that MAPC cells do not preferentially stimulate abnormal clonal cell proliferation (Figure 2C).

Generation and characterization of a murine MDS model by overexpression of the oncogene *EVII* in hematopoietic progenitor cells

Expression of *EVII* in hematopoietic progenitors is associated with the development of aggressive myeloid leukemia when it is inappropriately activated after chromosomal rearrangement [19,20]. It has previously been shown that overexpression of this oncogene in Lin⁻ BM cells can induce a low-grade, pancytopenic model of MDS in mice representative for an intrinsic defect in the HSC [20]. We used this MDS mouse model to investigate the effect of MAPC *in vivo*. Lin⁻ murine BM cells were transduced with a LV expressing *EVII* (*EVII*-IRES-GFP) and the mixed *EVII*⁺/GFP⁺ and *EVII*⁻/GFP⁻ population was transplanted in lethally irradiated (8.5 Gy) C57Bl/6 mice. Pancytopenia developed 9–15 months after BM transplantation (Figure 3A). When severe pancytopenia occurred the mice were sacrificed. *EVII* mice did not have splenomegaly, and the spleen retained a normal structure of white and red pulp without signs of extra-medullary hematopoiesis (data not shown). Morphologic analysis of BM and blood smears revealed cells with dysplastic features such as hyper-lobular, dysplastic megakaryocytes in the BM and polychromasia in blood of *EVII* mice, which is also a hallmark for human MDS (Figure 3B).

Quantitative reverse transcriptase PCR analysis on total BM cells (data not shown) and blood (Figure 3C) confirmed the high level overexpression of *EVII* ($P < 0.0001$) in *EVII* mice compared with controls. The total number of BM cells and percentage of Lin⁻ cells in BM-MNCs was similar in *EVII* compared with control mice (data not shown). As for human MDS BM, BM of *EVII* mice contained fewer CFU-GM and BFU-E colonies compared with control mice (Figure 3D). Likewise, BM of *EVII* mice contained significantly fewer LTC-ICs (Figure 3E).

We also performed secondary transplantations using BM from mice grafted with *EVII* transduced Lin⁻ cells at the time when the mice became cytopenic. Mice transplanted with a 3:1 or 5:1 ratio of *EVII* diseased and wild type BM developed one or more cytopenias after 6–10 months with a median survival time of 8 months. This was significantly faster than in primary grafted mice who had a median survival time of 14.2 months ($P < 0.0001$) (Figure 3F). Once again, high levels of *EVII* mRNA were observed in secondary transplanted *EVII* mice (data not shown). Signs of leukemic transformation were not detected in secondary transplanted animals. As in primary transplanted mice, CFCs and LTC-IC frequencies were decreased in secondary transplanted *EVII* mice (data not shown).

Biodistribution and persistence of MAPC cells in a murine MDS model

MAPC cells were transduced with the EF1 α -FlucNIS LV and injected in *EVII* mice (*EVII*-transduced; at the time they exhibited cytopenias) or empty vector (control transduced) control mice ($0.7\text{--}1 \times 10^6$ cells). BLI indicated that most cells were initially trapped in the lungs and gradually disappeared after 1 week in both diseased and control mice. However, in the diseased animals, but not control mice, a robust BLI signal was observed in the femurs and pelvic region up to 8 days after infusion of MAPC cells ($P < 0.05$) (Figure 4A).

Presence of MAPC cells in the femurs was confirmed by immunohistochemistry and quantitative PCR for the detection of human DNA. The presence of hNIS-expressing cells was confirmed mainly in EVI1 mice where numbers of positive cells increased until day 4. Control mice showed fewer positive cells than diseased mice. However, on day 4, an increased hNIS staining could be observed (Figure 5A). Human DNA in femurs of EVI1 mice was maximal on day 4, decreasing afterward but still detectable on day 21. In control mice, the maximal amount of human DNA was observed on day 4, although lower than in diseased animals and human DNA disappeared by day 8 (Figure 5B).

Therapeutic effect of MAPC cells in a murine MDS model

Finally, we used the murine MDS model to study the *in vivo* effect of MAPC cell therapy on BM failure. The frequency of CFCs and LTC-ICs in BM of *EVI1*-transduced mice infused with 0.75×10^6 MAPC cells (intravenous) was enumerated and compared with the frequency of hematopoietic progenitors in mice grafted with control-transduced and untransduced BM-MNCs. A significant increase in BFU-E and CFU-GM numbers was observed 8–21 days after transplantation of MAPC cells in treated *EVI1*-transduced mice to levels near those in control mice. Likewise, the LTC-IC frequency was significantly increased in MAPC cell treated *EVI1*-transduced mice to levels similar to those of animals transplanted with control-transduced BM-MNCs, but lower than mice grafted with untransduced BM (Figure 5C).

Discussion

The aim of this study was to evaluate the effect of MAPC cells as a cell-based therapy in MDS. Therapeutic options are still limited for the majority of MDS patients, and there is a need for new, innovative treatment strategies.

By using BM samples from untreated MDS patients, we demonstrated a positive effect of MAPC cells on the hematopoietic capacity of MDS BM cells. This effect was seen both when MAPC cells were cultured in contact or non-contact with MDS hematopoietic progenitor cells, indicating the importance of one or more soluble factors produced by MAPC cells. Regular expansion medium containing fetal bovine serum and growth factors (epidermal and platelet-derived growth factor) did not affect MDS CFC or LTC-IC proliferation. We also demonstrated that MAPC cells can provide stromal support for MDS-derived hematopoietic cells. We know from previous microarray data that human MAPCs express hematopoiesis-supporting cytokines *SCF*, *CXCL5*, *IL-8*, *IL-11*, *TGF-1 β* , *VEGF*, *ANGPT1* and *SPARC* [11], but future studies will need to investigate the secretome of MAPC cells when co-cultured with MDS BM cells to determine the responsible factor(s) for hematopoietic improvement seen in MDS. A general secretome analysis of human MAPC cells revealed the presence of molecules that are involved in extracellular matrix regulation and angiogenesis as well as secretion of cytokines that influence cells from the innate and adaptive immune system in their recruitment and effector function [21]. Further examination of the transcriptional and secretome changes induced in PBMCs by MAPC cells (provided in a transwell insert) after activation of T cells, support the hypothesis that MAPC cells block PBMC proliferation by cell cycle arrest via tryptophan depletion [22]. It was also

shown that cell surface expression of human leucocyte antigen-DR is minimal on MAPC cells exposed to activated PBMCs, providing a possible explanation of the immune-privileged MAPC cell status.

Similar hematopoiesis supporting activity and associated cytokine production was reported for MSC populations [23,24], and some of these are extensively studied in clinical trials to improve hematopoietic recovery after BM transplantation or for the *ex vivo* expansion of CD34⁺ progenitor cells [25–29].

Some studies have suggested that BM-derived MSCs contribute to the tumor cell niche and support tumor persistence [30]. Decreased stromal support, together with mounting evidence of a deregulated immune system, are believed to play an important role in the pathogenesis of MDS [31]. However, it has also been hypothesized that the immune/inflammatory suppression of hematopoiesis in MDS may serve to inhibit the proliferation of the malignant clonal cells and that removal of such inhibition may lead to progression to acute myeloid leukemia [32]. Because the observed positive effect of MAPC cells was mainly on myeloid progenitor cells, it was important to evaluate their effect on abnormal growth of the MDS clone. Using FISH analysis on BM cells derived from patients with a known genetic abnormality, we demonstrated that MAPC cell treatment did not preferentially increase the growth of abnormal clones, which is important from a safety point of view.

A second part of the study focused on the evaluation of MAPC cell therapy *in vivo*. For this, we successfully established and characterized a murine model for MDS by overexpressing the oncogene *EVII*. As previously published [19], we also observed aberrations in PB cell counts, blood and BM morphology and colony formation capacity. Therefore, this model can be considered a robust model to study MDS *in vivo*. Moreover, secondary transplantations could speed up the disease onset by 6 months on average, which substantially facilitated our studies to test MAPC cell therapy. We also used two types of controls (mice transplanted with untransduced Lin[−] cells or with empty vector transduced Lin[−] cells) and noticed a difference in the number of LTC-IC, being higher in untransduced transplanted controls, although not significantly.

Biodistribution studies demonstrated that MAPC cells, when administered intravenously, have a first-pass effect in the lung and subsequently migrate toward the site of injury, being the BM. BLI was performed for 8 days, but cells can be detected up to 21 days with sensitive methods like PCR. Moreover, a significant improvement in the hematopoietic capacity of treated MDS mice was seen compared with untreated animals. Improvement in cytopenias after MSC treatment has also been seen in mouse models of acute radiation syndrome [33,34]. In addition, MSC infusion in systemic lupus erythematosus patients with refractory cytopenias resulted in increased blood cell counts, which might have been associated with the reconstitution of regulatory T and Th17 cells [35].

However, the exact *in vivo* mode of action of the MAPC cell therapy in the murine MDS model has yet to be elucidated. On the basis of pilot experiments, a 1:1 ratio for MAPC:HSC was used *in vitro*, but because only a small minority of MAPC cells reached the BM, it is possible that the mode of action *in vivo* is not dependent on MAPC cells reaching the BM

niche. For example, MAPC cell interactions with inflammatory cells in other organs such as the lung or perhaps spleen may affect pathophysiological processes in other tissues, as has been demonstrated for MAPC cells injected in a model of traumatic brain injury [36].

In summary, we show here that MAPC cells can provide stromal support and enhance hematopoiesis in patient-derived hematopoietic cells, presumably through the production of soluble factor(s) *in vitro*. Our results also indicate that MAPC cell treatment does not preferentially enhance proliferation of the malignant MDS clone. Furthermore, intravenously injected MAPC cells can migrate to the place of injury and restore the hematopoietic function in a murine model of MDS.

These studies represent an initial proof-of-concept for a beneficial effect of MAPC cell treatment in patients with severe and symptomatic cytopenias underlying MDS and should form the basis for future clinical trials in humans.

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References

- [1]. Ornstein MC, Mukherjee S, Sekeres MA. More is better: combination therapies for myelodysplastic syndromes. *Best Pract Res Clin Haematol*. 2015; 28:22–31. [PubMed: 25659727]
- [2]. Sekeres MA. The epidemiology of myelodysplastic syndromes. *Hematol Oncol Clin North Am*. 2010; 24:287–94. [PubMed: 20359626]
- [3]. Nimer SD. Myelodysplastic syndromes. *Blood*. 2008; 111(10):4841–51. [PubMed: 18467609]
- [4]. Delforge M, Raets V, Van Duppen V, Vandenberghe P, Boogaerts M. CD34+ marrow progenitors from MDS patients with high levels of intramedullary apoptosis have reduced expression of alpha4beta1 and alpha5beta1 integrins. *Leukemia*. 2005; 19:57–63. [PubMed: 15510209]
- [5]. Ma L, Ceuppens J, Kasran A, Delforge M, Boogaerts M, Vandenberghe P. Immature and mature monocyte-derived dendritic cells in myelodysplastic syndromes of subtypes refractory anemia or refractory anemia with ringed sideroblasts display an altered cytokine profile. *Leuk Res*. 2007; 31:1373–82. [PubMed: 17188353]
- [6]. Ma L, Delforge M, van Duppen V, Verhoef G, Emanuel B, Boogaerts M, et al. Circulating myeloid and lymphoid precursor dendritic cells are clonally involved in myelodysplastic syndromes. *Leukemia*. 2004; 18:1451–6. [PubMed: 15284864]
- [7]. Meers S, Kasran A, Boon L, Lemmens J, Ravot C, Boogaerts M, et al. Monocytes are activated in patients with myelodysplastic syndromes and can contribute to bone marrow failure through CD40-CD40L interactions with T helper cells. *Leukemia*. 2007; 21:2411–19. [PubMed: 17805323]
- [8]. Meers S, Vandenberghe P, Boogaerts M, Verhoef G, Delforge M. The clinical significance of activated lymphocytes in patients with myelodysplastic syndromes: a single centre study of 131 patients. *Leuk Res*. 2008; 32:1026–35. [PubMed: 18006057]
- [9]. Oh IH, Kwon KR. Concise review: multiple niches for hematopoietic stem cell regulations. *Stem Cells*. 2010; 28:1243–9. [PubMed: 20517982]
- [10]. Tehranchi R, Woll PS, Anderson K, Buza-Vidas N, Mizukami T, Mead AJ, et al. Persistent malignant stem cells in del(5q) myelodysplasia in remission. *N Engl J Med*. 2010; 363:1025–37. [PubMed: 20825315]

- [11]. Roobrouck VD, Clavel C, Jacobs SA, Ulloa-Montoya F, Crippa S, Sohni A, et al. Differentiation potential of human postnatal mesenchymal stem cells, mesoangioblasts, and multipotent adult progenitor cells reflected in their transcriptome and partially influenced by the culture conditions. *Stem Cells*. 2011; 29:871–82. [PubMed: 21433224]
- [12]. Jacobs SA, Pinxteren J, Roobrouck VD, Luyckx A, van't Hof W, Deans R, et al. Human multipotent adult progenitor cells are nonimmunogenic and exert potent immunomodulatory effects on alloreactive T-cell responses. *Cell Transplant*. 2013; 22:1915–28. [PubMed: 23031260]
- [13]. Reading JL, Yang JH, Sabbah S, Skowera A, Knight RR, Pinxteren J, et al. Clinical-grade multipotent adult progenitor cells durably control pathogenic T cell responses in human models of transplantation and autoimmunity. *J Immunol*. 2013; 190:4542–52. [PubMed: 23547116]
- [14]. Jacobs SA, Roobrouck VD, Verfaillie CM, Van Gool SW. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. *Immunol Cell Biol*. 2013; 91:32–9. [PubMed: 23295415]
- [15]. Jacobs SA, Plessers J, Pinxteren J, Roobrouck VD, Verfaillie CM, Van Gool SW. Mutual interaction between human multipotent adult progenitor cells and NK cells. *Cell Transplant*. 2014; 23:1099–110. [PubMed: 23562064]
- [16]. Maziarz RT, Devos T, Bachier CR, Goldstein SC, Leis JF, Devine SM, et al. Single and multiple dose MultiStem (multipotent adult progenitor cell) therapy prophylaxis of acute graft-versus-host disease in myeloablative allogeneic hematopoietic cell transplantation: a phase 1 trial. *Biol Blood Marrow Transplant*. 2015; 21:720–8. [PubMed: 25555450]
- [17]. Wolfs E, Holvoet B, Gijssbers R, Casteels C, Roberts SJ, Struys T, et al. Optimization of multimodal imaging of mesenchymal stem cells using the human sodium iodide symporter for PET and Cerenkov luminescence imaging. *PLoS ONE*. 2014; 9:e94833.
- [18]. Sato T, Kim S, Selleri C, Young NS, Maciejewski JP. Measurement of secondary colony formation after 5 weeks in long-term cultures in patients with myelodysplastic syndrome. *Leukemia*. 1998; 12:1187–94. [PubMed: 9697872]
- [19]. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, van Putten WL, Valk PJ, van der Poel-van de Luytgaarde S, Hack R, et al. High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. *Blood*. 2003; 101:837–45. [PubMed: 12393383]
- [20]. Buonamici S, Li D, Chi Y, Zhao R, Wang X, Brace L, et al. EVI1 induces myelodysplastic syndrome in mice. *J Clin Invest*. 2004; 114:713–19. [PubMed: 15343390]
- [21]. Burrows GG, Van't Hof W, Newell LF, Reddy A, Wilmarth PA, David LL, et al. Dissection of the human multipotent adult progenitor cell secretome by proteomic analysis. *Stem Cells Transl Med*. 2013; 2:745–57. [PubMed: 23981727]
- [22]. Burrows GG, Van't Hof W, Reddy AP, Wilmarth PA, David LL, Raber A, et al. Solution-phase crosstalk and regulatory interactions between multipotent adult progenitor cells and peripheral blood mononuclear cells. *Stem Cells Transl Med*. 2015; 4:1436–49. [PubMed: 26494783]
- [23]. Kogler G, Radke TF, Lefort A, Sensken S, Fischer J, Sorg RV, et al. Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells. *Exp Hematol*. 2005; 33:573–83. [PubMed: 15850835]
- [24]. Majumdar MK, Thiede MA, Haynesworth SE, Bruder SP, Gerson SL. Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. *J Hematother Stem Cell Res*. 2000; 9:841–8. [PubMed: 11177595]
- [25]. Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol*. 2000; 18:307–16. [PubMed: 10637244]
- [26]. Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant*. 2005; 11:389–98. [PubMed: 15846293]

- [27]. Le Blanc K, Samuelsson H, Gustafsson B, Remberger M, Sundberg B, Arvidson J, et al. Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. *Leukemia*. 2007; 21:1733–8. [PubMed: 17541394]
- [28]. Liu K, Chen Y, Zeng Y, Xu L, Liu D, Chen H, et al. Coinfusion of mesenchymal stromal cells facilitates platelet recovery without increasing leukemia recurrence in haploidentical hematopoietic stem cell transplantation: a randomized, controlled clinical study. *Stem Cells Dev*. 2011; 20:1679–85. [PubMed: 21142788]
- [29]. de Lima M, McNiece I, Robinson SN, Munsell M, Eapen M, Horowitz M, et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. *N Engl J Med*. 2012; 367:2305–15. [PubMed: 23234514]
- [30]. Quante M, Tu SP, Tomita H, Gonda T, Wang SS, Takashi S, et al. Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. *Cancer Cell*. 2011; 19:257–72. [PubMed: 21316604]
- [31]. Ganan-Gomez I, Wei Y, Starczynowski DT, Colla S, Yang H, Cabrero-Calvo M, et al. Deregulation of innate immune and inflammatory signaling in myelodysplastic syndromes. *Leukemia*. 2015; 29:1458–69. [PubMed: 25761935]
- [32]. Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. *Blood*. 2006; 108:2509–19. [PubMed: 16778145]
- [33]. Hu KX, Sun QY, Guo M, Ai HS. The radiation protection and therapy effects of mesenchymal stem cells in mice with acute radiation injury. *Br J Radiol*. 2010; 83:52–8. [PubMed: 20139249]
- [34]. Lange C, Brunswig-Spickenheier B, Cappallo-Obermann H, Eggert K, Gehling UM, Rudolph C, et al. Radiation rescue: mesenchymal stromal cells protect from lethal irradiation. *PLoS ONE*. 2011; 6:e14486. [PubMed: 21245929]
- [35]. Li X, Wang D, Liang J, Zhang H, Sun L. Mesenchymal SCT ameliorates refractory cytopenia in patients with systemic lupus erythematosus. *Bone Marrow Transplant*. 2013; 48:544–50. [PubMed: 23064040]
- [36]. Walker PA, Shah SK, Jimenez F, Gerber MH, Xue H, Cutrone R, et al. Intravenous multipotent adult progenitor cell therapy for traumatic brain injury: preserving the blood brain barrier via an interaction with splenocytes. *Exp Neurol*. 2010; 225:341–52. [PubMed: 20637752]

Figure 1.

Decreased CFU-GM and LTC-IC frequency in MDS BM can be partially corrected by addition of MAPC cells. The BM cellularity, defined as the number of BM-MNCs per milliliter aspirate, and the percentage of CD34⁺ cells in patients (n = 20), healthy age-matched (n = 8) and young (n = 10) controls. (A) Left: CFC assay = the number of BFU-E and CFU-GM per 15 000 BM-MNCs in patients (n = 10) and healthy controls (n = 8), in triplicate. Right: LTC-IC assay = total number of LTC-ICs per 15 000 CD34⁺ cells in patients (n = 16) and healthy controls (n = 7). (B) Effect of MAPC cells on CFC frequency: the number of BFU-E and CFU-GM per 15 000 BM-MNCs in controls (n = 8) and patients (n = 10). Cells were plated in triplicate with or without the addition of MAPC cells in a transwell insert above the culture. Results from studies without MAPC cells are the same as in Figure 1B. Effect of MAPC cells on LTC-IC frequency: total number of LTC-ICs per 15 000 CD34⁺ cells from patients (n = 16) and healthy controls (n = 7) with or without the addition of MAPC cells (non-contact culture). (C) Morphology of colonies from the CFC and LTC-IC assay in patients with or without the addition of MAPC cells (non-contact culture).

Figure 2.

Beneficial MAPC cell effect is in part mediated by the secretion of soluble factor(s) in co-cultures and in part by supporting CFC growth. LTC-IC assay with patient CD34⁺ cells (15 000) in the presence of a second MAPC donor (non-contact) (n = 10), MAPC cells given intermittently (non-contact IM) in a transwell insert above the culture (n = 10), MAPC cells given in contact (n = 10) or MAPC medium alone without any cells given in a transwell insert above the culture (n = 9). (A) LTC-IC assay with patient CD34⁺ cells using conventional AFT024 feeders (n = 7) or MAPC feeders (n = 7 for MAPC donor 1, n = 6 for

MAPC donor 2). A representative colony grown on MAPC feeders is shown in the right panel. (B) FISH analysis of known cytogenetic abnormalities in four MDS patients. Graphs represent the percentage of abnormal cells in progeny of LTC-ICs cultured with or without MAPC cells for the known genetic abnormalities.

Figure 3.

Characterization of a murine MDS model. Blood counts in control and in EVI1 mice. Red blood cells (RBC; $\times 10^6$ cells/ μ L), white blood cells (WBC; $\times 10^3$ cells/ μ L) and platelets ($\times 10^3$ cells/ μ L). (A) BM and blood smears of control mice demonstrated a normal morphology. Hyperlobular, dysplastic megakaryocytes (black arrow) were found in the BM of EVI1 mice. Polychromasia (black arrow) was found in the blood of EVI1 mice. (B) *EVI1* mRNA expression in blood of EVI1 ($n = 16$, 7 months) and control mice (control-transduced BM-MNCs) ($n = 8$, 5–6 months); and untransduced BM-MNCs ($n = 10$, 7–9 months) BM grafted. (C) CFC assay: the number of BFU-E and CFU-GM in EVI1 ($n = 19$) and control mice (which received control-transduced or untransduced BM-MNCs; both $n = 10$). (D) LTC-IC assay: the frequency ($\times 1000$) of LTC-IC in EVI1 ($n = 12$) and control mice (which received control-transduced or untransduced BM-MNC; both $n = 9$). (E) Kaplan-Meier curves for primary ($n = 18$) and secondary ($n = 16$) transplanted animals (median survival time: 14.2 versus 8 months, $P < 0.0001$). Tx, treatment.

Figure 4.

Biodistribution of MAPC cells in a murine MDS model. (A) BLI on days 1, 2, 4, 6 and 8 after MAPC cell administration in the tail vein of mice grafted with EVI1-transduced and control-transduced BM-MNCs, at the time the EVI1-transduced mice developed pancytopenia. (B) Quantification of the BLI signal in lungs, femur and pelvic region in EVI1-transduced ($n = 6$) and control-transduced ($n = 3$) BM-MNCs, at the time the EVI1-transduced mice developed pancytopenia. $*P < 0.05$.

Figure 5.

In vivo persistence and therapeutic effect of MAPC cells. (A) hNIS staining of BM sections of MAPC-treated mice that received EVI1-transduced and control-transduced BM-MNCs, at the time the EVI1-transduced mice developed pancytopenia. (B) Human DNA quantification in the femur of MAPC-treated mice that received EVI1-transduced and control-transduced BM-MNCs, at the time the EVI1-transduced mice developed pancytopenia ($n = 5$, technical replicates). (C) CFC and LTC-IC assay in BM of mice that received EVI1-transduced with and without MAPC cells at the time the EVI1-transduced mice developed pancytopenia ($n = 11$; $0.7\text{--}1.0 \times 10^6$ cells injected intravenously). Data were compared with untreated EVI1 mice that received control-transduced ($n = 12$) or untransduced ($n = 9$) BM-MNCs.

Table I

Primer sequences.

Primer	Sequence
<i>EVII</i> Fw	GGACTACAAAGACGATGACGACAAGCT
<i>EVII</i> Rv	GCTGAGTGAGGAGTACTGCATGGAAACT
<i>gapdh</i> Fw	AAGGGCTCATGACCACAGTC
<i>gapdh</i> Rv	GGATGCAGGGATGATGTTCT
Human chromosome 17 Fw	ATGCTGATGTCTGGGTAGGGTG
Human chromosome 17 Rv	TGAGTCAGGAGCCAGCGTATG

Table II

Patient characteristics.

ID	Sex	MDS subtype
1	M	Cytopenia of unknown origin
2	M	RAEB-2
3	V	Cytopenia of unknown origin
4	M	RCMD with fibrosis
5	M	RARS
6	M	RARS
7	M	Cytopenia of unknown origin
8	M	RA
9	V	Cytopenia of unknown origin
10	M	RARS
11	V	Megakaryocytic dysplasia
12	M	RA
13	M	RAEB-2
14	V	RCMD
15	V	RA
16	M	RARS
17	M	RCMD
18	V	RA
19	V	5q-syndrome
20	M	RARS

Patients were diagnosed with cytopenia of unknown origin or MDS according to the different subcategories of the World Health Organization classification.