Serine Protease Inhibitor Kunitz-Type 2 Is Downregulated in Myelodysplastic Syndromes and Modulates Cell–Cell Adhesion

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Myelodysplastic syndromes (MDS) are clonal disorders involving hematopoietic stem cells (HSC) characterized by ineffective hematopoiesis. In addition to HSC defects, a defective hematopoiesis supporting capacity of mesenchymal stromal cells (MSCs) in the microenvironment niche has been implicated in MDS pathophysiology. The interaction between the dysfunctional MSCs MDS and HSC regulates diverse adhesion-related processes, such as progenitor cell survival, proliferation, differentiation, and self-renewal. As previously reported, a microarray analysis identified serine protease inhibitor kunitz-type 2 (*SPINT2*), an inhibitor of hepatocyte growth factor (*HGF*) activation, to be downregulated in MSCs from MDS patients. To define the role of *SPINT2* in MDS hematopoietic microenvironment, an analysis of the effect of *SPINT2* silencing in MSCs was carried out. We herein reported significantly lower levels of *SPINT2* whereas *HGF* was expressed at higher levels in MSCs from MDS patients compared with healthy controls. *SPINT2* underexpression results in an increased expression, production, and secretion of HGF and stromal cell-derived factor 1 (*SDF-1*) by MSCs. An increased adhesion of normal HSC or malignant cells onto MSCs silenced for *SPINT2* was also observed. The altered MSCs adhesion in *SPINT2*-knockdown cells was correlated with increased CD49b and CD49d expression and with a decrease in CD49e expression. Our results suggest that the *SPINT2* underexpression in the MSC from MDS patients is probably involved in the adhesion of progenitors to the bone marrow niche, through an increased HGF and SDF-1 signaling pathway.

Background

MYELODYSPLASTIC SYNDROMES (MDS) correspond to a heterogeneous group of clonal disorders involving hematopoietic stem cells (HSC) characterized by peripheral blood cytopenias, ineffective hematopoiesis, and an increased risk of progressing toward acute myeloid leukemia (AML) [1]. There is evidence that in addition to HSC defects, an important role is also played by the hematopoietic bone marrow (BM) microenvironment niche. This niche is responsible for mediating the direct cell contact with HSC and for supporting the selection of neoplastic hematopoietic clones [2]. Alterations in this BM microenvironment, such as abnormal interactions with HSC or malignant clones, deficient production of hematopoietic growth factors, and aberrant release of cytokines, contribute to the pathogenesis of

MDS [3]. The BM microenvironment is composed of several cell types, including mesenchymal stromal cells (MSCs), which are key components in supporting self-renewal and proliferation of hematopoietic cell progenitors [4]. Numerous studies have demonstrated the morphological and functional alterations in MSCs from MDS patients [5], such as modifications in gene expression and in cytokine secretion [6].

Our group recently identified new possible target genes involved in MDS pathophysiology through the microarray analysis of MSCs from MDS patients [7]. Among the genes identified, an interesting underexpressed gene found was serine protease inhibitor kunitz-type 2 (*SPINT2*), encoding a transmembrane protein called hepatocyte growth factor activator (HGFA) inhibitor 2 (HAI-2). HAI-2 protein inhibits the enzyme HGFA, responsible for the conversion of hepatocyte growth factor (HGF) into its active form [8]. HGF

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is a polypeptide secreted by MSCs that acts as a multifunctional cytokine, regulating adhesion, growth, and survival of hematopoietic cells [9]. The levels of serum HGF cytokine are significantly augmented in MDS patients, and are considered a predictor of survival [10].

SPINT2 is underexpressed in some types of solid cancers and is correlated with the prognostic and progression of these cancers [11]; however, the functional role of *SPINT2* in MDS and myeloid cells is still unknown. In this study, we assessed the expression levels of *SPINT2* and *HGF* in normal and dysplastic MSCs in order to understand the functional role of *SPINT2* in MDS MSCs and determine whether this gene expression correlated with a malignant progression in MDS.

Methods

Patients and controls

BM aspirates were collected according to institutional guidelines from healthy donors and untreated MDS patients. For gene expression analysis, MSCs were isolated from the BM aspirates of 6 healthy donors and 15 untreated MDS patients (11 low risk and 4 high risk). For adhesion assays, CD34⁺ cells were obtained from the peripheral blood of three healthy donors. Assignment to different groups was decided according to the 2008 World Health Organization classification. For analysis of total BM, BM aspirates were collected from 22 healthy donors and 48 untreated patients (27 low risk and 21 high risk) (Table 1).

This study was approved by the Ethics Committee of the University of Campinas. All healthy donors and patients provided informed written consent. Patients with a confirmed diagnosis of MDS, untreated at the time of sample

collection, and who had attended the outpatient clinic from 2005 and 2013 were included in the study.

CD34⁺ cell and MSCs selection

The BM mononuclear cells were isolated by Ficoll-Hypaque Plus density-gradient centrifugation (GE Healthcare, Uppsala, Sweden) and labeled with CD34 MicroBeads (Miltenyi Biotec, Auburn, CA). CD34⁺ cells were isolated by MIDI-MACS immunoaffinity columns (Miltenyi Biotec) and purity was determined by flow cytometry (at least 90%), using anti-CD34 antibody conjugated to allophycocyanin (APC; Becton Dickinson, San Jose, CA).

The mononuclear cells without CD34⁺ cells were plated onto Iscove's modified Dulbecco's media (IMDM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 10% horse serum or onto Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% FBS. The supernatant containing nonadherent cells was removed weekly and replaced with fresh supplemented medium. When the monolayer was established (90% confluence), cells were trypsinized and plated under the same conditions. After three replatings, a homogeneous cell population was obtained and MSCs were evaluated by flow cytometry for the absence of CD31, CD34, CD45, CD68, and HLA-DR antigens and the presence of CD73, CD90, and CD105.

Cell culture and reagent chemicals

HS5 and HS-27a cell lines, which are known to be representative human MSCs, and U937 cells were obtained from ATCC (Manassas, VA). The P39 cell line was kindly provided by Prof. Dr. Eva Hellström-Lindberg (Department of Medicine, Division of Hematology, Karolinska University, Stockholm, Sweden). Cells were cultured in Roswell Park Memorial Institute medium-1640 (RPMI; Sigma) containing 10% FBS, 2 mM glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. All cells were maintained at 5% CO₂ and 37°C temperature in CO₂ incubator. Recombinant human HGF was purchased from PeproTech (Rocky Hill, NJ).

Quantitative polymerase chain reaction

All samples were assayed with cDNA (RevertAid H Minus First Strand cDNA Synthesis Kit; MBI Fermentas, St. Leon-Rot, Germany), SYBR Green Master Mix PCR (MBI Fermentas), and specific primers in the ABI 7500 Sequence Detection System (Applied-Biosystem, Foster City, CA). The relative gene expression was calculated using the equation $2^{-\Delta\Delta CT}$ [12]. Control was performed for each primer pair. Amplification specificity was verified using a dissociation curve at the end of each run. Three replicates were run on the same plate for each sample. The following primers were used: *HGF*, 5'-TGACTCCGAACAGGATTCTTTCA-3' and 5'-GCAGGGCTGGCAGGAGTT-3'; stromal cell-derived factor 1 (*SDF-1*), 5'-GAGCTACAGATGCCCATGC-3' and 5'-CTTTA GCTTCGGGTCAATGC-3'; *SPINT2*, 5'-TCTGTTTCTCTGG GAGGTAGGA-3' and 5'-CGATCAGCGAGGAAACAAC-3'; and hypoxanthine guanine phosphoribosyl transferase (*HPRT*), 5'-GAACGTCTTGCTCGAGATGTGA-3' and 5'-TCCAGCAGGTCAGCAAAGAAT-3'.

TABLE 1. PATIENT CHARACTERISTICS

	MSCs sample number	Total BM sample number
Controls	6	22
Gender		
Male/Female	4/2	16/6
Age (years), median (range)	36 (27–47)	33 (16–49)
MDS patients	15	48
Gender		
Male/Female	11/4	32/16
Age (years), median (range)	69 (34–77)	67 (16–87)
FAB		
Low-risk (RA/RARS)	3/8	18/9
High-risk (RAEB/RAEBt)	4/0	19/2
WHO		
Low-risk (RCUD/RCMD/RARS)	0/3/8	1/17/9
High-risk (RAEB-1/RAEB-2)	1/3	12/9

MSCs, mesenchymal stromal cell; BM, bone marrow; MDS, myelodysplastic syndrome; FAB, French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess of blasts; RAEBt, refractory anemia with excess of blasts in transformation; WHO, World Health Organization; RCUD, refractory cytopenia with unilineage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; RAEB-1, refractory anemia with excess blast-1; RAEB-2, refractory anemia with excess blast-2.

Western blot

Equal amounts of protein were electrophoresed on sodium dodecyl sulfate polyacrylamide gels under reducing conditions. Nitrocellulose membranes were then immunostained with anti-HAI-2 antibody (ab128926) from Abcam (Cambridge, MA) or anti-Actin antibody (sc-1616) from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes were visualized with chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and exposed to Amersham Hyperfilm ECL film (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Quantitative analyses of the optical intensities of protein bands were determined with Un-Scan-It Gel 6.1 (Silk Scientific, Inc., Orem, UT) and normalized by actin.

Transduction of lentivirus

HS5 and HS-27a cells were transduced with lentivirus-mediated shRNA nonspecific control (sc-108080) or lentivirus-mediated shRNA targeting *SPINT2* (sc-39556-V) from Santa Cruz Biotechnology and, namely, shControl and shSPINT2 cells, respectively. Briefly, mesenchymal cell lines were seeded into six-well plates at 1.5×10^5 cells/well, grown overnight, and transduced with lentiviral vectors at multiplicity of infection equal to 0.5 in a minimal volume of medium containing 6 $\mu\text{g/mL}$ of polybrene (Sigma-Aldrich, St. Louis, MO). The transduced cells were selected for 10–15 days using puromycin (0.50 and 1.20 $\mu\text{g/mL}$, respectively, to HS5 and HS-27a cells; Santa Cruz, CA). Puromycin-resistant cells were expanded and analyzed for cytokine production, cell-to-cell adhesion, and integrin expression profile.

Quantification of HGF and SDF-1 secretion

Culture supernatants from shControl and shSPINT2 cells were assayed for HGF and SDF-1 human cytokines using a Bio-Plex Pro Human Cytokine from Bio-Rad (Hercules, CA) after 0, 6, 12, 24, and 48 h of culture. Briefly, beads conjugated to the analyte-specific capture antibodies were incubated with cell supernatants or standard-curve samples in 96-well plates. A biotinylated detector antibody was added to each well, followed by Streptavidin. Samples were analyzed in a Luminex 100 instrument (Bio-Rad). The concentration of each cytokine was determined from the standard curve, which was generated using a five-parameter algorithm.

Coculture of HS5 or HS-27a MSCs and hematopoietic cells

Stromal feeder layers from nontransduced, shControl, and shSPINT2 cells were seeded in 24-well culture plates (1×10^4 cells/well for coculture with P39 and U937 cells or 3×10^4 cells/well for coculture with CD34⁺ cells) in serum-free RPMI plus bovine serum albumin (BSA) and incubated for 48 h at 37°C. After this period, nonadherent cells were seeded onto stromal cell monolayers (4×10^5 P39 or U937 cells/well or 3×10^5 CD34⁺ cells/well), allowing to adhere in serum-free RPMI plus BSA for 30 min for P39 or U937 or 24 h for CD34⁺ cells. Nonadherent cells were removed, and

the adherent cells were collected by vigorously pipetting cold phosphate-buffered saline (PBS). CD45 expression was used to distinguish hematopoietic cells from MSCs as only hematopoietic cells, including P39 and U937 cells, express this CD45 antigen. The CD34 marker was used to distinguish CD34⁺ cells from stromal cells. Cocultured cells were labeled with a CD45 or CD34 antibody conjugated to APC (BD Biosciences, San Jose, CA), monitored by flow cytometry using an FACS Calibur (BD Biosciences). The analysis was carried out using the FACS Diva software (BD Biosciences). Percentages of cells expressing each marker were determined out of a total 10,000 events.

Adhesion molecule profile

Stromal feeder layers from nontransduced, shControl, and shSPINT2 cells were seeded onto 24-well culture plates (1×10^5 cells/well) in serum-free RPMI plus BSA and incubated for 48 h at 37°C. In rescue-type experiments, recombinant human HGF (50 ng/mL) was added to the cultures mentioned earlier. The cells were then trypsinized, washed with PBS, and resuspended in PBS. Cells were incubated for 30 min with monoclonal antibodies against the following adhesion receptor: CD49b, CD49d, and CD49e (BD Biosciences). Cells were then evaluated by flow cytometer in an FACS Calibur and analyzed using the FACS Diva software. A total of 10,000 events were collected per sample. The distribution histogram was used to determine the geometric mean of the fluorescence intensity (MFI) for each antibody tested. The degree of positivity for each tested surface adhesion receptor was expressed as a numerical MFI of the positively stained cells.

Statistical analysis

Statistical analysis was performed using GraphPad Prism5 software (GraphPad, San Diego, CA). Data were expressed as the median [minimum–maximum]. For comparisons, an appropriate Mann–Whitney test or analysis of variance test was used. Spearman correlation analysis was used for ranking correlation tests. The values of $P < 0.05$ were considered as statistically significant. All experiments were repeated a minimum of three independent times.

Results

Decreased *SPINT2* expression and increased HGF expression in MDS cells compared with normal cells

The first step of our study comprised the evaluation of *SPINT2* and *HGF* mRNA expression in MSCs from primary cultures generated from MDS and healthy BM aspirates.

The age-adjusted expression of *SPINT2* in MSCs and total BM cells from MDS patients compared with cells from healthy donors were analyzed by quantitative polymerase chain reaction (qPCR). *SPINT2* mRNA expression was significantly decreased in MSCs from MDS patients compared with MSCs from healthy donors (0.35 [0.01–2.63] vs. 0.89 [0.46–1.59], $P < 0.05$) (Fig. 1A). There was no significant difference in *SPINT2* mRNA expression between low-risk MDS MSCs and high-risk MDS MSCs (0.32 [0.07–1.07] vs.

FIG. 1. Serine protease inhibitor kunitz-type 2 (*SPINT2*) and hepatocyte growth factor (*HGF*) expression in normal and myelodysplastic mesenchymal stromal cells (MSCs). (A) Age-adjusted *SPINT2* mRNA expression was significantly lower in MSCs from myelodysplastic syndrome (MDS) patients compared with healthy donors. (B) Age-adjusted *HGF* mRNA expression was significantly higher in MSCs from MDS patients as compared with healthy donors. (C) Horizontal lines represent median values; Mann–Whitney and analysis of variance tests were used as nonparametric test. (C) Correlation analysis between *SPINT2* expression and *HGF* expression in MSCs. *P* and *r* values are indicated; Spearman correlation test.

0.43 [0.01–2.63], $P=0.58$). There was also a significant decrease of *SPINT2* expression in total BM cells from MDS patients compared with cells from healthy donors (0.42 [0.01–2.77] vs. 0.91 [0.14–4.79], $P<0.05$).

As *SPINT2* inhibits HGFA, responsible for the conversion of HGF into its active form, *HGF* expression was evaluated. The age-adjusted *HGF* mRNA expression was significantly increased in MSCs from MDS patients compared with MSCs from healthy donors (4.76 [0.91–29.04]

vs. 1.11 [0.49–3.01], $P<0.001$) (Fig. 1B). There was no significant difference in *HGF* mRNA expression between low-risk MDS MSCs and high-risk MDS MSCs (4.76 [1.44–29.04] vs. 4.40 [0.91–4.99], $P=0.40$).

The correlation of *SPINT2* and *HGF* expression was also analyzed. *SPINT2* mRNA expression was negatively correlated with *HGF* mRNA expression ($r = -0.60$, $P<0.01$, Spearman test). Thus, when *SPINT2* expression was low, the expression of *HGF* was high (Fig. 1C).

FIG. 2. Lentivirus-mediated shRNA targeting *SPINT2* effectively silenced *SPINT2* in HS5 and HS-27a cell lines. Quantitative expression of *SPINT2* mRNA in shSPINT2 cells relative to the shControl cells in HS5 (A) and HS-27a (B) cell lines. Lentivirus-mediated *SPINT2* shRNA effectively silenced *SPINT2* in both MSCs lines. mRNA expression levels of *SPINT2* were normalized by hypoxanthine guanine phosphoribosyl transferase (*HPRT*) endogenous control, as indicated. Results were analyzed using $2^{-\Delta\Delta CT}$. Experiments were performed in triplicate. Western blot analysis of shControl and shSPINT2 total cell extracts of HS5 (C) and HS-27a (D) cell lines. The membrane was blotted with antibodies against *SPINT2* protein/HGFA inhibitor 2 (HAI-2, 34 kDa) or actin (42 kDa), as a control for equal sample loading, and developed with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). *P* values are indicated.

SPINT2 silencing resulted in an increased HGF and SDF-1 cytokine secretion

As the next step, we decided to further evaluate the role of *SPINT2* inhibition on MSCs. To examine the effects of *SPINT2* on the MSCs model, HS5 and HS-27a cell lines were stably transduced with lentivirus-mediated shRNA targeting *SPINT2* or an appropriate control. Nontransduced cells were also included. After selection by antibiotic, mRNA and protein levels of *SPINT2* were determined by qPCR and western blot, respectively. Significant reductions in *SPINT2* mRNA (by $74 \pm 3\%$, $P < 0.0001$, and $69 \pm 3\%$, $P < 0.001$, for HS5 and HS27a cell lines, respectively) and protein (by $58 \pm 7\%$, $P < 0.001$, and $64 \pm 8\%$, $P < 0.001$, for HS5 and HS27a cell lines, respectively) levels were observed in shSPINT2 cells when compared with shControl cells (Fig. 2).

To determine whether alterations in the *SPINT2* expression of MSCs contributed to the production of the cytokines HGF and SDF-1, we measured the levels of these cytokines in culture supernatants from shControl and shSPINT2 cells after 0, 6, 12, 24, and 48 h. Under our culture conditions, an increase in the secretion levels of HGF and SDF-1 was observed in shSPINT2 cells when compared with shControl cells. In HS5 cells, *SPINT2* inhibition resulted in a significant higher secretion of HGF after 24 and 48 h of culture (Fig. 3A) and a significant higher SDF-1 secretion after 48 h of culture (Fig. 3B). In HS-27a cells, *SPINT2* inhibition resulted in a significant higher level of HGF secretion after 24 and 48 h of culture (Fig. 3C) and a significant higher secretion of SDF-1 after 48 h of culture (Fig. 3D).

Downregulation of SPINT2 increased the adhesion of MSCs to CD34⁺ or P39 or U937 cells

As HGF has been reported to induce hematopoietic cell adhesion to MSCs, we investigated the influence of HGF secretion from cells, silenced or not for the *SPINT2* gene, on the adhesion to myeloid cell lines, P39 and U937. In both HS5 and HS-27a cell lines, an increased adherence between shSPINT2 cells and human myeloid cell lines was observed when compared with shControl cells or nontransduced cells, as follows: HS5+P39 cells: 46.35% [40.50%–60.10%] versus 31.45% [20.20%–38.20%] or 33.50% [32.00%–38.00%], $P < 0.01$; HS5+U937 cells: 83.30% [73.50%–90.20%] versus 57.30 [55.40%–63.00%] or 59.80 [54.70%–70.90%], $P < 0.01$; HS27a+P39: 38.35% [37.20%–39.50%] versus 25.10% [23.40%–31.70%] or 28.50 [26.00%–33.00%], $P < 0.01$; and HS27a+U937: 90.10% [87.50%–93.50%] versus 76.05% [51.10%–80.90%] or 71.95% [70.20%–72.60%], $P < 0.01$ (Fig. 4).

The levels of cytokine secretion were higher in HS-27a cell line compared with HS5 cells. Therefore, we selected the HS-27a cell line to analyze the adherence between CD34⁺ cells and shControl or shSPINT2 cells. *SPINT2* gene silencing induced a significant increase in CD34⁺ cell adherence to MSCs when compared with shControl cells or nontransduced cells (67.60% [53.10%–73.60%] vs. 53.70% [46.40%–67.60%] or 53.80 [47.60%–54.60%], respectively, $P < 0.05$) (Fig. 4E).

HGF and SDF-1 cytokines secreted by MSCs can regulate some cellular processes, such as adhesion; therefore, a correlation analysis using Spearman test was carried out between HGF or SDF1 secretion levels and the percentage of adherent

FIG. 3. Increase in HGF and stromal cell-derived factor 1 (SDF-1) secretion in supernatants of shSPINT2 cells (HS5 and HS-27a cells) measured by the Luminex-based Bio-Plex assay. Time-course analysis of HGF (A–C) and SDF-1 (B–D) secreted by shControl and shSPINT2 HS5 and HS-27a cell lines, as indicated. Data shown are the mean $\pm$ standard deviation of three independent experiments. Statistical analysis: Mann-Whitney test. P values are indicated.

FIG. 4. SPINT2 silencing induces hematopoietic cell and pluripotent stem cell adhesion onto MSCs. P39 (A–C) and U937 (B–D) cell lines were added to a monolayer of nontransduced, shControl, or shSPINT2 HS5 or HS-27a cells and allowed to adhere for 30 min, as indicated. The adherent cells were measured by flow cytometry using CD45-allophycocyanin (APC) and analyzed as percentage of total cells. (E) Primary CD34⁺ cells from healthy donors were added to a monolayer of nontransduced, shControl, or shSPINT2 HS-27a cells and allowed to adhere for 24 h. The adherent cells were measured by flow cytometry using CD34-APC and analyzed as percentage of total cells. Percentages of cells expressing each marker were determined out of a total of 10,000 events. Ø—nontransduced cells; shControl—cells transduced with lentivirus-mediated shRNA nonspecific control; shSPINT2—cells transduced with lentivirus-mediated shRNA targeting *SPINT2*. Values are mean $\pm$ standard deviation of six different experiments. Statistical analysis: Mann–Whitney test. *P* values are indicated.

CD45 or CD34 cells. Since the levels of cytokine secretion were higher in HS-27a cells compared with HS5 cell line, we selected the HS-27a cell line to carry out these analyses. The results showed a positive and significant correlation between HGF and CD45- or CD34-positive cells as well as between SDF-1 and CD45- or CD34-positive cells (Fig. 5).

Downregulation of *SPINT2* and high secretion of HGF and SDF-1 alters the alpha integrin expression profile

To further analyze the mechanism of how *SPINT2* inhibition with consequent high HGF secretion acts on cellular adhesion, we examined the expression of CD49b (α_2), CD49d (α_4), and CD49e (α_5) integrins, as these molecules are known to mediate cell adhesion in HS-27a cell line. shSPINT2 cells induced a significant increase in the expression of CD49b (1022.0 [836.0–1235.0] vs. 856.0 [759.0–967.0] or 818.0 [801.0–853.0], $P < 0.01$) and CD49d (1676.0 [1062.0–1980.0] vs. 1177.0 [761.0–1832.0] or 939.0 [811.0–1425.0], $P < 0.01$) (Fig. 6A, B, respectively) and a significant decrease in the expression of CD49e (4276.0 [3124.0–5177.0] vs. 6750.0 [5621.0–7672.0] or 6049.0

[5579.0–6765.0], $P < 0.01$) (Fig. 6C) when compared with shControl cells or nontransduced cells, respectively. The cell number expressing CD49b (17.10% [11.30%–23.10%] vs. 11.50% [4.20%–16.30%] or 11.30% [2.10%–13.00%], $P < 0.05$) was higher in shSPINT2 cells when compared with shControl cells or with nontransduced cells, respectively (Fig. 6D). No difference in the cell number expressing CD49d and CD49e was observed between shSPINT2 and shControl cells or nontransduced cells (Fig. 6E, F).

The treatment with recombinant HGF induces SDF-1 expression and alters the alpha integrin expression profile

To verify whether high HGF secretion caused by *SPINT2* inhibition is responsible for alterations in SDF-1 and alpha integrin expression, we treated nontransduced, shControl, and shSPINT2 cells with active recombinant HGF. The screening of *SDF-1* was carried out by real-time PCR since HGF upregulates *SDF-1* mRNA expression [13]. The treatment of nontransduced cells and shControl cells with recombinant HGF resulted in an increased expression of *SDF-1* mRNA similar to the shSPINT2 cells (Fig. 7).

FIG. 5. Correlation of HGF or SDF-1 and CD45- or CD34-positive cells (%). (A) Correlation analysis between HGF secretion and % CD45 of P39 cell adhesion onto HS-27a. (B) Correlation analysis between HGF secretion and % CD45 of U937 cell adhesion onto HS-27a. (C) Correlation analysis between HGF secretion and % CD34 of CD34⁺ cell adhesion onto HS-27a. (D) Correlation analysis between SDF-1 secretion and % CD45 of P39 cell adhesion onto HS-27a. (E) Correlation analysis between SDF-1 secretion and % CD45 of U937 cell adhesion onto HS-27a. (F) Correlation analysis between SDF-1 secretion and % CD34 of CD34⁺ cell adhesion onto HS-27a cells. P and r values are indicated; Spearman correlation test.

The treatment of shControl cells with recombinant HGF induced a significant increase in the expression of CD49b (1072.0 [932.0–1222.0] vs. 856.0 [759.0–967.0], $P < 0.05$) and CD49d (2298.0 [2016.0–2575.0] vs. 939.0 [811.0–1425.0], $P < 0.01$) (Fig. 6A, B, respectively) when compared with shControl cells. The treatment of nontransduced cells with recombinant HGF also induced a significant increase in the expression of CD49b (1052.0 [905.0–1199.0] vs. 818.0 [801.0–853.0], $P < 0.01$) and CD49d (2751.0 [1710.0–3751.0] vs. 939.0 [811.0–1425.0], $P < 0.01$) (Fig. 6A, B, respectively) when compared with nontransduced cells. A significant decrease in the expression of CD49e was observed in shControl cells with recombinant HGF when compared with shControl cells (1940.0 [1525.0–2692.0] vs. 6750.0 [5621.0–7672.0], $P < 0.01$) (Fig. 6C) and in nontransduced cells with recombinant HGF when compared with nontransduced cells (2139.0 [1651.0–2537.0] vs. 6049.0 [5579.0–6765.0], $P < 0.05$) (Fig. 6C). The cell number expressing CD49b was higher in cells treated with HGF when compared with nontreated cells (Fig. 6D). No difference in the cell number expressing CD49d and CD49e was observed between treated and nontreated cells (Fig. 6E, F). The treatment of nontransduced cells and shControl cells with recombinant HGF resulted in alpha integrin profile alterations similar to those of shSPINT2 cells.

Discussion

The mechanisms responsible for hematopoietic failure in MDS patients have not yet been fully elucidated. There is evidence that suggests that this failure is caused by an association between intrinsic abnormalities within the HSC compartment and defective mesenchymal stromal support mechanisms [6]. Recently, the BM microenvironment niche has received considerable attention in MDS pathophysiology since components of the cell niche, mainly MSCs, are defective in supporting normal hematopoiesis in MDS [14], mostly in myelopoiesis [15], and can induce dysplasia in hematopoietic precursors in vitro [16]. MSCs facilitate the self-renewal, survival, differentiation, and proliferation of hematopoietic cells through direct (cell–cell) and indirect (cytokine secretion) contact [17,18]. MSCs constitutively secrete a large number of cytokines and alterations in their levels can also serve as a safe haven for malignant hematopoietic cells, resulting in an important modification in MDS clones and offering protection against chemotherapeutic agents [19].

In an attempt to better understand the abnormalities in MSCs MDS, our group analyzed a 44k combined intron-exon oligoarray platform to determine protein-coding transcript expression profiles in MSCs from MDS patients and from

FIG. 6. SPINT2 inhibition and cells treated with recombinant HGF induce alteration in alpha integrin expression in MSCs. The expression of the integrins CD49b, CD49d, and CD49e was analyzed by flow cytometry. (A) CD49b expression. (B) CD49d expression. (C) CD49e expression. (D) Percentage of total cells expressing CD49b. (E) Percentage of total cells expressing CD49d. (F) Percentage of total cells expressing CD49e. The distribution histogram was used to determine the geometric mean of the fluorescence intensity (MFI). The degree of positivity for each tested surface adhesion receptor was expressed as MFI. Percentages of cells expressing each marker were determined out of a total of 10,000 events. Ø—nontransduced cells; Ø+HGF—nontransduced cells treated with recombinant HGF; shControl—cells transduced with lentivirus-mediated shRNA nonspecific control; shControl+HGF—cells transduced with lentivirus-mediated shRNA nonspecific control treated with recombinant HGF; shSPINT2—cells transduced with lentivirus-mediated shRNA targeting SPINT2; shSPINT2+HGF—cells transduced with lentivirus-mediated shRNA targeting SPINT2 treated with recombinant HGF. Values are mean $\pm$ standard deviation of six different experiments. Statistical analysis: Mann–Whitney test. *P* values are indicated.

healthy donors. Several differentially expressed transcripts were identified and classified according to their molecular and cellular functions, including cell motility, DNA replication, protein phosphorylation, and protein transport [7]. Among the transcripts identified, an interesting under-expressed gene, *SPINT2*, presented a role as a tumor suppressor and appeared to be related to the poor prognosis of some solid cancers [20]. To validate and confirm the array results, we investigated herein, the expression of *SPINT2* by qPCR in MSCs obtained from 15 MDS patients and 6 healthy donors. Some of these samples were plated in IMDM plus FBS and horse serum, whereas others were plated in DMEM plus FBS. Despite these differences in culture, the analysis of *SPINT2* mRNA expression showed similar levels and statistical significance, indicating that the medium did not interfere in the expression of this gene. Our results demonstrated that *SPINT2* was underexpressed in MSCs from MDS patients when compared with healthy donor cells, suggesting that this gene may be involved in MDS microenvironment abnormalities. Similarly, an un-

derexpression of *SPINT2* in total BM was observed. In the attempt to identify the BM cells expressing *SPINT2*, we also analyzed the *SPINT2* expression in HSC from BM. Interestingly, we detected no *SPINT2* expression in HSC either from MDS patients or from healthy donors. Thus, we suggest that *SPINT2*/HAI-2 may be specifically produced by MSCs and, despite MSCs representing only 0.001%–0.01% of the nucleated BM cells, in total BM this gene expression can be detected [21].

SPINT2 gene is implicated in the dysregulation of HGF signaling pathway [22] by encoding the HAI-2 transmembrane protein. HAI-2 is responsible for the inhibition of HGFA enzyme, which catalyzes HGF activation through the conversion of HGF into its active form [8,23]. HGF is constitutively produced by MSCs and has mitogenic and antiapoptotic effects on diverse cell types [24]. In the light of these findings, we hypothesized that the decrease in *SPINT2* expression in MDS could result in an increased HGF expression, production, and secretion. Therefore, we further analyzed, using qPCR, the *HGF* mRNA expression

FIG. 7. SPINT2 inhibition and cells treated with recombinant HGF induce *SDF-1* expression in MSCs. The expression of *SDF-1* mRNA was analyzed by real-time polymerase chain reaction. Ø—nontransduced cells; Ø+HGF—nontransduced cells treated with recombinant HGF; shControl—cells transduced with lentivirus-mediated shRNA nonspecific control; shControl+HGF—cells transduced with lentivirus-mediated shRNA nonspecific control treated with recombinant HGF; shSPINT2—cells transduced with lentivirus-mediated shRNA targeting SPINT2; shSPINT2+HGF—cells transduced with lentivirus-mediated shRNA targeting SPINT2 treated with recombinant HGF. Values are mean $\pm$ standard deviation of three different experiments. Statistical analysis: Mann–Whitney test. *P* values are indicated.

in MSCs from MDS patients and healthy donors. An increased expression of *HGF* mRNA was found in MSCs from MDS patients when compared with healthy donor cells. Our results are in accordance with Keith et al. who showed a significantly higher expression of *HGF* mRNA in BM specimens of MDS and de novo AML compared with controls [25]. Moreover, recent studies demonstrated that the serum levels of HGF cytokine in MDS patients are significantly increased and dependent on the severity of MDS [10,26].

To elucidate the role of *SPINT2* on the secretion of HGF in MSCs, we used a lentiviral vector delivering shRNA specific to human *SPINT2* gene to inhibit its expression in two distinct MSCs lines, HS5 and HS-27a. One major problem of using shRNAs in experimentation is the possibility of off-target effects. Off-target activity can complicate the interpretation of phenotypic effects and can potentially lead to unwanted or unexpected toxicities. A nonspecific shRNA (negative control) allows discriminating actual knockdown from general effects of transfection, including the toxicity due spinoculation, liposome, or antibiotic selection [27]. However, an untreated cell control is also important to evaluate general cell culture conditions since nonspecific shRNA can alter some cell properties and invalidate the analysis. Hence, to confirm the specificity of interference RNA results, a pool of three shRNAs targeting three different regions on *SPINT2* gene was used. In addition, shRNA that targets irrelevant genes was used as a negative control and nontransduced cells were used as a control for modification of cell properties. The results showed a significant increase in the secretion of HGF after 24 and 48 h of culture in the cells transduced with shSPINT2 when compared with shControl cells in both MSCs lines. Thereby, decreased levels of SPINT2 were unable to inhibit HGFA enzyme, resulting in the conversion of pro-HGF in

the active heterodimer of the HGF cytokine that was expressed, produced, and secreted in higher levels by MSCs silenced for SPINT2.

HGF is a cytokine capable of maintaining the mesenchymal microenvironment niche through promoting hematopoiesis by inducing constitutive production of some other cytokines, such as interleukin (IL)-11, SDF-1, and stem cell factor (SCF) [28]. Among these factors, SDF-1 is an important chemoattractive cytokine implicated in the pathophysiology of MDS [29] since in vitro studies have shown increased SDF-1 levels in the BM and plasma of MDS patients when compared with healthy donors [30]. This cytokine, responsible for the regulation of nonadherent hematopoietic cells [31], is required for hematopoietic cell migration, adhesion, and BM retention [32]. Increased secretion of SDF-1 is also important due to its regulation of the traffic of normal and malignant hematopoietic cells to the mesenchymal stromal microenvironment niche, affecting self-renewal, homing, and proliferation of hematopoietic cells [33]. In abnormal matured effector cells of MDS patients with high expression levels of SDF-1 receptor (CXCR4), an increased homing to the BM is observed [34], possibly resulting in an increased leukemic cell survival. Thus, we decided to analyze the SDF-1 secretion in supernatant of shSPINT2 and shControl cells. The result showed a significant increase in the secretion of SDF-1 in shSPINT2 cells compared with shControl cells in both MSCs lines. Likewise, treating shControl cells with recombinant HGF resulted in an improved *SDF-1* mRNA expression to levels similar to those of shSPINT2 cells. Therefore, the increased secretion of HGF of shSPINT2 cells probably results in an autocrine regulator, which induces the production and secretion of SDF-1 by MSCs themselves. Several studies have shown that there is an imbalance in cytokine levels on the MDS microenvironment niche [16], which can be related to the prognosis of MDS patients [35,36].

A difference in the quantity of bioactive HGF and SDF-1 secretions by HS5 and HS-27a was observed. The alteration in HGF secretion can be explained by the fact that HS27a cells express high levels of niche-associated cytokines, such as HGF, to support primitive hematopoietic precursors in specialized areas and HS5 cells express low levels of niche-associated ligands, but secretes high levels of factors, such as IL-1, IL-6, and granulocyte colony stimulating factor (G-CSF), which drive hematopoietic precursors to differentiate and proliferate [37].

The external cytokine stimuli provided by MDS MSCs can regulate some cellular processes, such as adhesion, survival, and proliferation [1,38]. Further, cell-to-cell interaction and adhesion among mesenchymal stromal microenvironment niche and HSC are essential to maintain stem cell characteristics and contribute to abnormal HSC growth and maturation [5]. Matsuda-Hashii et al. showed that cultures treated with HGF neutralizing antibody inhibited MSCs adhesion [28]. Likewise, SDF-1 was described as being responsible for promoting ML1 and U937 cell adhesion to the HS5 and HS27a MSCs lines by stimulating the PI3K pathway [39]. Since high levels of HGF and SDF-1 cytokines have been reported to induce cell adhesion, we investigated the static adhesion between shControl or shSPINT2 MSCs with pluripotent stem cells or leukemia cell lines, using a coculture system. An increase in the

adherence between shSPINT2 cells and CD34⁺ cells or P39 cells or U937 cells was observed. Therefore, these results support the hypothesis that the improved secretion of HGF and SDF-1 cytokines can be associated with increased adhesion of cells inhibited for *SPINT2* gene as occurs in MDS patients.

Numerous cytokines are well known to modulate cell-to-cell and cell-to-extracellular matrix adhesion by influencing the number and/or the ligand binding affinity of CD49, an alpha integrin family member [40]. For example, Trusolino et al. showed that HGF could promote cell adhesion and invasiveness in epithelial cells by increasing the regulation of CD49 integrin avidity to their specific ligands [41]. In this way, we analyzed the adhesion profile of the integrins CD49b, CD49d, and CD49e on cells inhibited or not for the *SPINT2* gene. These integrins are known to play an important role in the interaction among HSC and BM microenvironment niche and to be essential for controlling hematopoiesis [42]. CD49d is a bidirectional signaling molecule that mediates the interaction between hematopoietic and microenvironmental cells and acts as a receptor for soluble signaling factors [43]. Higher CD49d expression significantly enhances HSC attachment and homing to MSCs [44] and is involved in the retention of normal HSC and leukemic blasts within the BM microenvironment, possibly modulating chemotherapy response [45]. The increased CD49d expression observed after SPINT2 inhibition could enable a better interaction among MSCs and HSC or leukemia cells, improving attachment, retention, self-renewal, and homing of these cells to the microenvironment niche. On the other hand, CD49e integrin is responsible for the interaction with the extracellular matrix and is abundantly expressed on the cell surface of MSCs [28]. Interestingly, MSCs growth defects were significantly correlated with decreases in CD49e expression [2]. Our results showed a decrease in the level of this CD49e integrin expression in shSPINT2 cells, as observed by Aanei et al. in MSCs from MDS patients [2], indicating that the SPINT2 underexpression could result in a lower affinity between MSCs and extracellular matrix, contributing to the abnormalities in MSCs MDS. Moreover, the inhibition of MSCs adhesion to extracellular matrix components by HGF neutralizing antibody was mediated through the activation of the CD49e integrin [28]. In addition, the activation of another integrin, CD49b, has a critical role in the BM-niche microenvironment preventing megakaryocyte development to platelet formation [46]. Improved CD49b expression in shSPINT2 cells may result in abnormalities in nonadherent cell differentiation, increasing the immature cell number in BM environment. Interestingly, the shControl cells treated with recombinant HGF showed the same alpha integrin expression profile as shSPINT2 cells. Taken together, SPINT2 silencing alters molecule receptor adhesion, probably by improving HGF secretion, which results in an increased expression of the integrin responsible for cell-to-cell adhesion and reducing the expression of an integrin that preferentially mediates cell interactions to extracellular matrix.

In view of these data, we propose that the underexpression of *SPINT2* gene in MDS results in an increased expression, production, and secretion of HGF cytokine with consequent autocrine-increased secretion of SDF-1 by the MSCs MDS. This alteration in the HGF and SDF-1 secretions causes an increased adherence between MSCs MDS

and pluripotent stem cells or leukemia cells, contributing to the maintenance of the properties of these cells, including self-renewal, homing, survival, and proliferation. The alterations in SPINT2/HAI-2 expression in MSCs from MDS patients may participate in the maintenance of abnormal pluripotent stem cells and malignant clones in MDS, probably contributing to its pathophysiology. These findings open a door to future investigation.

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Author Disclosure Statement

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