

Osteogenic Potential of Mesenchymal Stromal Cells Contributes to Primary Myelofibrosis

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Abstract

Primary myelofibrosis is a myeloproliferative neoplasm that is a precursor to myeloid leukemia. Dysmegakaryopoiesis and extramedullary hematopoiesis characterize primary myelofibrosis, which is also associated with bone marrow stromal alterations marked by fibrosis, neoangiogenesis, and osteomyelosclerosis. In particular, contributions to primary myelofibrosis from mesenchymal stromal cells (MSC) have been suggested by mouse studies, but evidence in humans remains lacking. In this study, we show that bone marrow MSCs from primary myelofibrosis patients exhibit unique molecular and functional abnormalities distinct from other myeloproliferative neoplasms and these abnormalities are maintained stably *ex vivo* in the absence of leukemic cells. Primary myelofibrosis-MSC overexpressed heparin-binding cytokines, including proinflammatory TGF β 1 and

osteogenic BMP-2, as well as glycosaminoglycans such as heparan sulfate and chondroitin sulfate. Transcriptome and functional analyses revealed alterations in MSC differentiation characterized by an increased osteogenic potential and a TGF β 1 signaling signature. Accordingly, phospho-Smad2 levels were intrinsically increased in primary myelofibrosis-MSC along with enhanced expression of the master bone regulator RUNX2, while inhibition of the endogenous TGF β 1 receptor TGF β R1 impaired osteogenic differentiation in these MSCs. Taken together, our results define the source of a critical osteogenic function in primary myelofibrosis that supports its pathophysiology, suggesting that combined targeting of both the hematopoietic and stromal cell compartments in primary myelofibrosis patients may heighten therapeutic efficacy. *Cancer Res*; 75(22); 4753–65. ©2015 AACR.

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Introduction

According to the 2008 WHO classification, primary myelofibrosis belongs to Philadelphia negative myeloproliferative neoplasms. Together with polycythemia vera and essential thrombocytopenia, primary myelofibrosis shares features of myeloproliferative diseases that are a clonal amplification of hematopoietic stem cells (HSC). Primary myelofibrosis is characterized by a shortened life expectancy and an alteration of bone marrow stroma as shown by myelofibrosis, neoangiogenesis, and osteosclerosis (1). Despite new leukemic cell targeted therapies, primary myelofibrosis is still regarded as an incurable disease except for rare young patients who are successful recipients of allogeneic stem cell transplantation. Such inefficiency is likely due to an incomplete understanding of its pathogenesis and especially of the role of the bone marrow stroma in the pathologic process. Actually, whereas current knowledge of hematopoietic cell alterations, and especially of the role of mutations including *Jak2*^{V617F} in the myeloproliferative process, partially explains the primary myelofibrosis pathogenesis, functional involvement of mesenchymal stromal cells remains poorly understood. Up to now, it is accepted that stromal changes are not inherent but are secondary to the cytokine production, especially TGF β 1, by clonal hematopoietic cells. However, recently, an increasing number of evidences strongly suggest the involvement of stromal alterations in the pathogenesis of primary myelofibrosis (2). First, medullar insufficiency is concomitant of an extramedullary hematopoiesis

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in spleen/liver, suggesting a plastic microenvironment that can be reinitialized to support hematopoiesis (3). Second, one of the primary myelofibrosis features is the increased circulating level of CD34⁺ HSCs, witnessing a perturbation of the CXCL12–CXCR4 axis and involving a deregulation of metalloprotease activity and integrin expression in the bone marrow (1). Third, we previously demonstrated phenotypic and functional alterations of fibroblasts isolated from the spleen of primary myelofibrosis patients (3). Finally, patient plasma reveals an inflammatory cytokine pattern that likely contributes to fibrosis, neoangiogenesis, and osteosclerosis (1). Finally, in mouse models, there are several evidences for stromal alterations inducing MPNs independently from hematopoietic cell abnormalities (4, 5).

Among stromal cells, mesenchymal stromal cells (MSC) are nonhematopoietic cells that possess multipotency, hematopoietic supporting, and immunomodulatory properties. They are main components of the bone marrow mesenchymal niches that participate in HSC regulation through interactions with endothelial cells, diffusible and environmental factors, as well as extracellular matrix (ECM) components (6). MSCs also function as a repository for precursor cells participating in bone development, maintenance, and remodeling (6). Whereas evidences for MSC abnormalities exist in murine models and are involved in bone stromal changes (7), proofs of acquired alterations are lacking in patients. Recently, Avanzini and colleagues reported genetic and functional aberrations of bone marrow MSCs in MPN patients and showed that MSCs exhibit decreased proliferative abilities as well as decreased osteogenic capacities (8). These findings are interesting but questioning as primary myelofibrosis is characterized by an osteosclerosis resulting from increased bone formation and as most of the patients analyzed in this report were under cytoreductive therapy. In the current manuscript, we reported that bone marrow MSCs from primary myelofibrosis patients, either at diagnosis or untreated, exhibit a persistent increase of their osteogenic abilities. This alteration is restricted to primary myelofibrosis as it is not observed in essential thrombocytopenia or polycythemia vera patients. More importantly, we showed that this alteration was maintained *ex vivo*, independently from any *in vivo* hematopoietic cell stimulation. Primary myelofibrosis-MSCs also display an altered secretome with an overproduction of

TGFβ1, BMP-2, and glycosaminoglycans (GAG) and a specific transcriptome signature in relation with a TGFβ1 impregnation. Functionally, inhibition of TGFβ R1 activation evidences the involvement of TGFβ in the osteogenic impairment of medullar MSCs from primary myelofibrosis patients. Altogether, our results identify for the first time an original and specific molecular signature of primary myelofibrosis-MSCs, suggesting their participation in the osteogenic deregulation observed in patients independently from any local stimulation by hematopoietic cells.

Patients and Methods

Patients and healthy volunteers

Twelve primary myelofibrosis patients at initial diagnosis and untreated at the time of the study, six age-matched at initial diagnosis essential thrombocytopenia/polycythemia vera patients and six age-matched healthy donors (HD) from our Orthopedic Unit (hip prosthesis surgery) were investigated (Table 1). Diagnosis was established by clinical features and bone marrow examination according to the WHO group (9). All samples were obtained with the informed consent of subjects according to Helsinki declaration.

Isolation and culture of bone marrow MSCs

MSCs isolated from patients were obtained from a fragment of bone marrow biopsy crushed in culture medium (MEM+10% FBS) and transferred in culture dish. After 3 days in 5% CO₂, nonadherent cells and bone fragments were removed. MSCs collected from HDs were isolated by adhesion to plastic. After 24 hours, nonadherent cells were removed. When 90% confluence was reached, cells were detached with Trypsin-EDTA (passage 1: P1). The culture medium was replaced twice a week. For proliferation assay, trypsinized cells were counted between passages 1 and 6; cell viability was assessed after Trypan Blue staining.

Phenotypic analysis

MSCs were washed with PBS containing BSA (Sigma-Aldrich), and labeled with CD90-PE (clone-5E10), CD73-PE (clone-AD2; BD-Biosciences), CD105-PE (clone-166707), CD45-FITC (clone-

Table 1. Characteristics of the patients enrolled in the study

Subject	Sex	Age (y)	Fibrosis grade	Platelets count G/L	Hb (g/L)	WBC G/L	CD34 circ. 10 ⁶ /L	Spleen size (cm)	JAK2 V617F	MPL W515L	CALR
MF1	M	61	MF3	409	9.3	12	107	>20	POS	NEG	NR
MF2	M	57	MF1	275	13.5	7	7	>20	POS	NEG	NR
MF3	M	68	MF2	875	12.7	16.5	8	NA	NEG	NA	NEG
MF4	M	49	MF3	266	15.2	6.9	8	18	POS	NEG	NR
MF5	M	47	MF2	97	10.6	10.1	296	NA	NEG	NEG	NEG
MF6	M	72	MF1	516	12.4	14	26	13	POS	NEG	NR
MF7	M	83	NA	NA	NA	NA	56	NA	NEG	NEG	NEG
MF8	M	51	MF2	625	12.8	9.8	1	13	POS	NEG	NR
MF9	M	51	MF1	530	12.1	8.9	345	16	NEG	NA	NEG
MF10	F	58	MF1	476	11	9.4	39	18	POS	NA	NR
MF11	F	55	MF1	142	11.7	3.5	29	18	NEG	NA	POS
MF12	M	73	MF2	106	9.7	16.3	NA	19	POS	NA	NR
PV1	M	84		211	21.1	9.3			POS	NR	NR
PV2	F	72		412	18.3	6.7			POS	NR	NR
PV3	M	63		284	19.3	8.7			POS	NR	NR
TE1	F	86		829	12.4	6.8			POS	NR	NR
TE2	M	35		1000	14.6	10.9			POS	NR	NR
TE3	M	67		968	13.5	12.5			POS	NR	NR

Abbreviations: CD34 circ., CD34-positive cells in peripheral blood; Hb, hemoglobinemia; MF, primary myelofibrosis; NA, not available; NR, nonrelevant; WBC, white blood cells; Y, years.

UCHL1), and HLA-DR-FITC (clone-L203; R&D systems) antibodies. Isotypes from same species were used as negative controls. Membrane fluorescence was quantified using Cellquest software on a FACScan system (Becton Dickinson; 10^5 live cells were analyzed).

JAK2/MPL/CALR mutation analysis

DNA from MSCs was purified using standard protocols. Detection of *JAK2*^{V617F} or *MPL*^{W515L/K} mutations was performed using an allele-specific RQ-PCR assay (Ipsogen Jak2 MutaQuant and MPLW515L/K MutaScreen, Qiagen). The *CALR* exon 9 mutations were screened by product sizing analysis and Sanger sequencing according to the methods described by Klampfl and colleagues (10).

Senescence assay

MSCs (5×10^4) from primary myelofibrosis patients were cultured in six-well dishes (48 hours). The β -galactosidase staining kit was used according to the manufacturer's specifications (Cell Signaling Technology). Senescent stained cells were scored by visualization using light microscopy (X40-100).

Hematopoietic assay

MSCs from primary myelofibrosis patients were irradiated (60 Gy) and cultured in long-term bone marrow culture (LTBMC) medium (MyeloCult H5100; Stem-Cell Technologies). Peripheral blood CD34⁺ cells from an immobilized single HD, purified as described (11), were layered onto the irradiated MSCs in LTBMC medium. Each week up to 5 weeks, cells were harvested and plated in methylcellulose medium consisting of 50 ng/mL SCF, 10 ng/mL IL3, 20 ng/mL GM-CSF, and 4 U/mL erythropoietin (Sigma-Aldrich). After 2 weeks, hematopoietic colonies greater than 50 cells were scored.

Cytokine array

Cells were cultured for 48 hours in MEM- α supplemented by 10% FBS, supernatants were removed and stored at -80°C prior the analysis, cells were counted. Quantitative measurement (pg/mL/ 10^5 cells) of IL1 β , IL1RA, IL6, IL8, IL10, IL12, IL13, IL15, EGF, bFGF, RANTES, PF4, VEGF, active TGF β 1, active TGF β 2, and TGF β 3, PDGF and Osteopontin was performed by Quantibody array (Raybiotech).

Bone marrow biopsy staining

Bone marrow trephine biopsies were obtained from our Pathology Unit. Specimens from primary myelofibrosis patients staged according to the WHO guidelines (9) were obtained prior any therapy. Retrieved tissues were fixed in formaldehyde, decalcified, and embedded in paraffin. Sections (5 μm) were analyzed in triplicate. GAG immunostaining was performed as described (12): AO4B08 antibody recognizes heparan sulfate (HS), and IO3H10 antibody reveals chondroitin sulfate (CS). Bound antibodies were detected with mouse antivesicular stomatitis virus (VSV) monoclonal antibody (P5D4) followed by a secondary antibody coupled to Alexa488 GAM (Life Technologies). Negative controls were stained with anti-VSV and Alexa488 GAM. Images were analyzed by ImageJ as described (13).

Glycosaminoglycan extraction and quantification

MSCs from primary myelofibrosis patients were cultivated in culture medium until confluence. A dry pellet (10^6 cells) was analyzed. After proteinase K (Merck) and DNase (Qiagen) treatment, proteins were precipitated and supernatants were cleared by chloroform washing followed by dialysis of the aqueous phase (Spectrum) against the extraction buffer and water. After freeze drying, identities of extracted GAGs were determined by specific

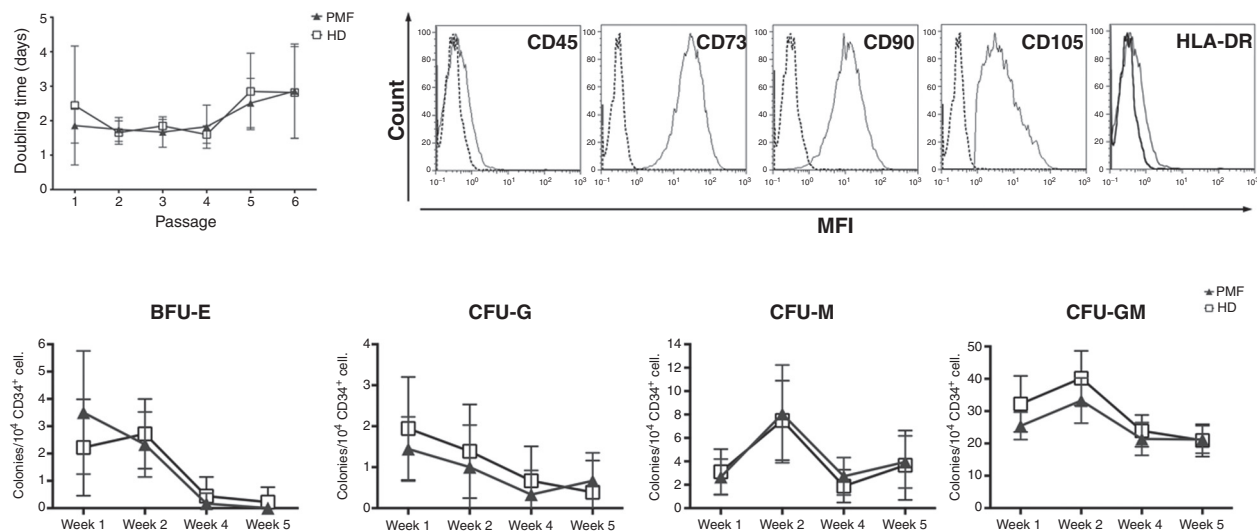
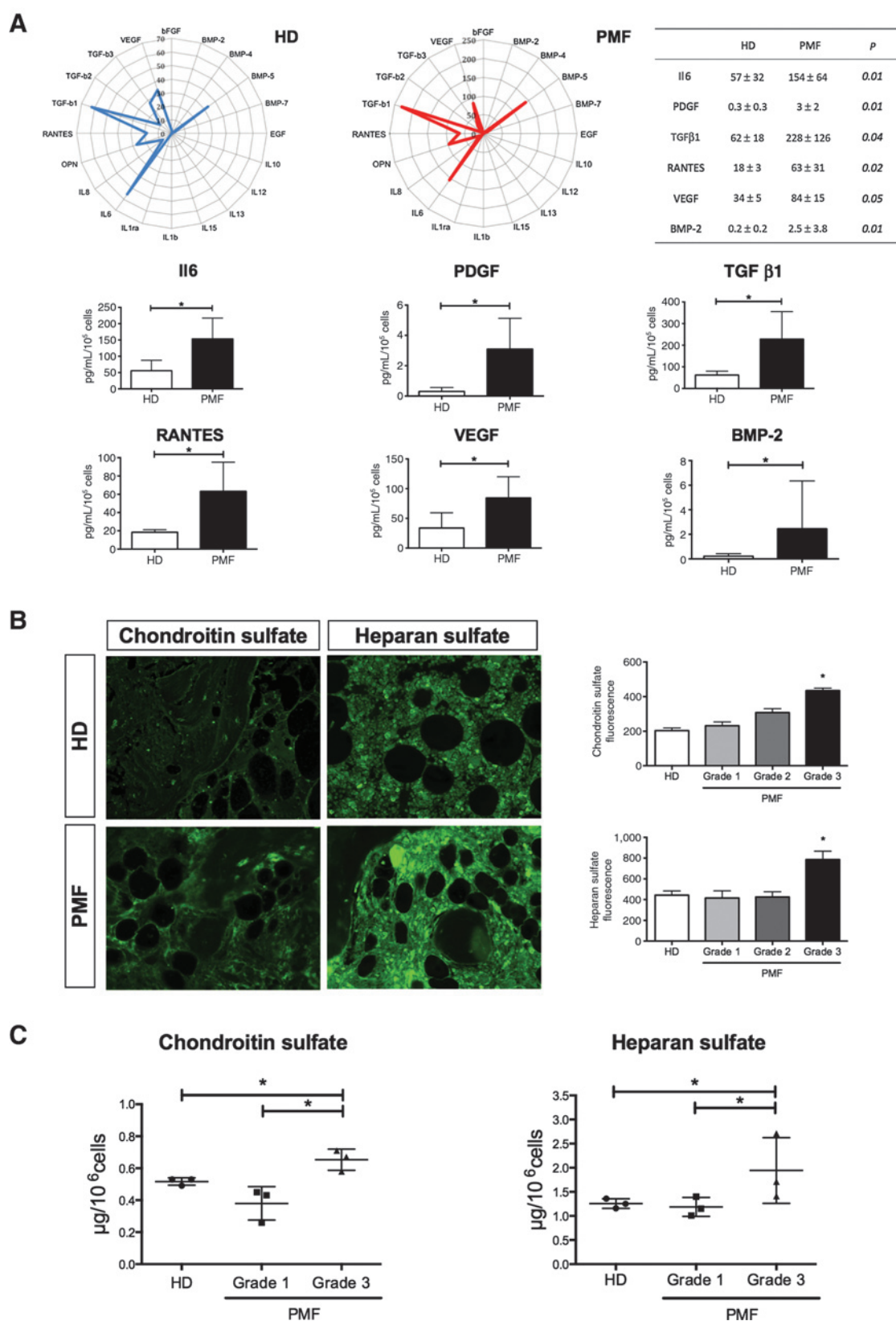


Figure 1.

Primary myelofibrosis (PMF)-MSCs exhibit similar proliferative capacity and long-term hematopoiesis support compared with healthy donors. A, doubling time of MSCs from primary myelofibrosis patients ($n = 4$) and healthy donors ($n = 8$) during successive passages (P) from P1 to P6. B, immunophenotype of primary myelofibrosis-MSCs analyzed by flow cytometry; control isotypes are presented in dashed line, markers in black, (MFI, median fluorescence intensity; $n = 6$). C, hematopoiesis support by MSCs from primary myelofibrosis ($n = 3$) and healthy donors ($n = 3$) in LTBMC; numbers of hematopoietic colonies BFU-E, CFU-G, CFU-M, and CFU-GM per 10^4 CD34⁺ cells from unmobilized peripheral blood. Results are expressed as mean \pm SD. White square, healthy donors; black triangle, primary myelofibrosis patients.

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digestion with chondroitinase ABC (Sigma-Aldrich) or by nitrous acid treatment and quantified according to the DMMB assay, based on sulfate groups complexation, as described (14).

Transcriptome analysis

RNAs were isolated using standard protocols and their quality was evaluated on an Agilent2100 Bioanalyzer (Agilent Technologies). T7-based amplification, labeling, and hybridization on Agilent Whole Human Genome Oligo Microarrays 4 × 44K was performed by Miltenyi Biotec using Agilent's technology. Normalized data are accessible on public database (GSE44426). Microarray analysis was performed by using gene set enrichment analysis (GSEA) from Broad Institute (15). To identify genes differentially and significantly regulated, expression ratios and corresponding *P* values were calculated and data were analyzed using MeV_4_5 Software. Unsupervised classification was performed on significant genes with metric of Pearson correlation with complete linkage. Principal component analysis allows discriminating patients and controls on the significant gene list in the first factorial map.

qRT-PCR analysis

Total RNAs were converted into cDNA using the Reverse Transcription kit (Applied Biosystems). cDNA (2 μL) was added to the Sybr Green amplification reaction mix (SG MasterMix, Roche Diagnostics) in a 20 μL final volume containing 10 pmol of each primer (Supplementary Table S1). Amplification cycles (*n* = 40) were performed on a Light Cycler 480 (Roche Diagnostics). Data were normalized on *β-actin*, *RPL38*, *RPL13a*, *18s*, and *GAPDH* housekeeping genes by a relative quantification, based on the $2^{-\Delta\Delta C_t}$ method.

In vivo MSC osteogenic assay

The use and care of animals were approved by the French Institutional Animal Care and Use Committee. Subconfluent MSCs from 3 HDs, 3 primary myelofibrosis, 3 essential thrombocytopenia, and 3 polycythemia vera patients were collected for subcutaneous implantation into the flanks of 10-week-old female nude mice. Implants (*n* = 3–12/group) were prepared by mixing sterile 80 to 200 μm particles (60% hydroxyapatite/40% βTCP) (Graftys), 100 μL of human plasma and 1×10^6 MSCs from HDs or from patients as described (16). Ten weeks after implantation, animals were sacrificed and the implants collected; one implant of each group was decalcified and the two others were not to preserve the mineralized bone formed. Implants were retrieved for histologic evaluation by HES and reticulin staining. Reticulin density was assessed by analysis of ImageJ recorded staining after skeletonization and area measurement.

In vitro MSC differentiation assay

Osteoblast, chondrocyte, and adipocyte differentiation abilities of expanded MSCs were determined as reported (17). Briefly,

MSCs were cultured ($2-3 \times 10^4/\text{cm}^2$) in osteogenic medium (α -MEM with 10 mmol/L β -glycerophosphate, 10^{-7} mol/L dexamethasone, 0.2 mmol/L ascorbic acid), adipogenic medium (DMEM with 10% FBS, 10^{-6} mol/L dexamethasone, insulin 10^{-2} mg/mL, indomethacin 2×10^{-1} mmol/L, 100 μg/mL 1-methyl-3-Isobutyl-xanthine), or chondrogenic medium (α -MEM, FBS 10%, TGFβ3) for up to 3 weeks. Differentiation into osteoblasts, adipocytes, and chondrocytes was evaluated by Von Kossa and Red Alizarin stains, oil-red staining, and Alcian blue staining, respectively. Red Alizarin quantification was performed as described (18).

TGFβ1 stimulation/inhibition and BMP inhibition

TGFβ1 was added in the medium at 10 ng/mL. Inhibition of TGFβ1 type I receptor ALK5 or BMP type I receptors ALK2, 3 and 6 was studied by adding 10 μmol/L of SB-431542 (19) or 1 μmol/L of LDN-193189 (Miltenyi Biotec; ref. 20), respectively, in differentiation medium that was changed twice a week up to 21 days.

Western blot analysis

Protein samples were generated as described (21). For each sample, 5 to 10 μg proteins were loaded and separated on SDS-PAGE. Membranes were probed with primary antibodies: anti-Runx-2 (Abcam) and anti-phospho-Smad2 (Sigma-Aldrich). Membranes were revealed with anti-rabbit IgG-peroxidase antibodies (Sigma-Aldrich). Immunoreactivity was detected by chemoluminescence (Roche Diagnostics). Spot signal intensity was quantified using Imaging (Bio-Rad). Data were expressed relative to an internal standard sample, as indicated in figure legends (21).

Statistical analysis

Results were expressed as mean ± SD. Statistical differences between patients and HDs were validated by unpaired *t* test with a significant *P* < 0.05. Statistical differences between conditions were validated by paired *t* test with significant *P* < 0.05. Fisher ANOVA test with two factors (samples and time of differentiation) was also used and *ad hoc* post-tests (Tukey) were calculated when required.

Results

Primary myelofibrosis-MSCs exhibit similar proliferative capacity and long-term hematopoiesis support compared with HD-MSCs

We first compared the biologic properties of MSCs obtained from the bone marrow of 12 primary myelofibrosis patients (Table 1) and of 6 HDs. One week after cultivation, adherent cells from primary myelofibrosis patients and HDs exhibited similar morphology and were able to form CFU-F colonies in similar proportions (7 ± 2 vs. $6 \pm 4/10^3$ cells). Figure 1A shows that the doubling time of primary myelofibrosis-MSCs was not significantly different from that of HDs. Observed β -galactosidase activity did not assess senescence of MSCs in primary myelofibrosis nor HDs at latest

Figure 2.

Primary myelofibrosis (PMF)-MSCs overproduce cytokines and glycosaminoglycans. A, cytokine production by MSCs. Results expressed as radar plot of all tested cytokines for controls (*n* = 4) in blue and primary myelofibrosis (*n* = 8) in red (note that scales are different), and representation of significantly overexpressed cytokines. Results expressed in pg/mL/ 10^5 cells, mean ± SD. B, staining of CS and HS of bone marrow section from HD and primary myelofibrosis patients; pictures show representative staining (magnification, ×100); quantification of fluorescence of CS and HS staining is represented in HD (*n* = 6) and in grade 1, 2, and 3 of fibrosis in primary myelofibrosis patients (*n* = 3 in each grade). C, CS and HS production by MSC from HD (*n* = 3) and grade 1 or 3 of fibrosis in primary myelofibrosis patients (*n* = 3 in each group). Results are expressed in μg/ 10^6 cells as mean ± SD. *, *P* < 0.05.

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passages (data not shown). MSCs from primary myelofibrosis, essential thrombocytopenia/polycythemia vera and HDs expressed similar phenotype ($CD73^+CD90^+CD105^+CD45^-HLADR^-$; Fig. 1B). To further confirm the absence of adherent $CD45^+$ monocytes/macrophages that could have been coisolated in our process, we estimated the content of contaminating hematopoietic cells in the preparations by analyzing JAK2/PML/CALR mutations in the culture pellets. We did not evidence mutations in the DNAs retrieved from cultures (data not shown). Long-term bone marrow cultures were performed to assess their hematopoietic support abilities for 1 to 5 weeks. Clonogenic assays performed each week (BFU-E, CFU-G, CFU-M, and CFU-GM; Supplementary Table S2) showed that MSCs from primary myelofibrosis patients ($n = 3$) and from HDs ($n = 3$) exhibited a similar capacity to support normal hematopoiesis (Fig. 1C).

Primary myelofibrosis-MSCs overproduce fibrogenic and inflammatory cytokines

Cytokine production by MSCs from 8 primary myelofibrosis patients and 4 HDs was studied (P3-P5) using quantitative ELISA. Figure 2A shows that MSCs from primary myelofibrosis patients overproduced cytokines such as IL6, PDGF, RANTES, BMP-2, and active TGF β 1. The absence of $CD45^+$ cells avoids a potential hematopoietic contamination that would participate in this overproduction. No BMP-4, BMP-7, EGF, IL10, IL12, IL13, IL15, and IL1 β were detected (<1 pg/mL/ 10^5 cells) and no significant difference was observed in the production of BMP-5, Osteopontin, PF4, and IL8 by MSCs isolated either from HDs or from primary myelofibrosis patients.

MSCs from fibrotic primary myelofibrosis patients express high levels of GAGs

We further analyzed the capacity of primary myelofibrosis-MSCs to produce sulfated GAGs reported to enhance the bioavailability of heparin binding protein (HBP), such as growth factors and chemokines. Bone marrow sections from patients ($n = 9$) and HDs ($n = 6$) were stained with antibodies against CS or HS GAG species and relative fluorescence level quantification were performed by specific ImageJ analysis (Fig. 2B). The level of CS or HS increased significantly with fibrosis grade in bone marrow of primary myelofibrosis patients: compared with HDs, primary myelofibrosis grade 3 exhibited higher level of CS and HS. We further investigated the HS and CS amount of GAGs produced *in vitro* by MSCs (P3-P5) from HDs and primary myelofibrosis patients with fibrosis grade 1 and 3 ($n = 3$ /group). MSCs from patients with grade 3 fibrosis exhibited a significantly higher production of HS and CS compared with patients with grade 1 fibrosis and to HDs, correlating immunostaining observations.

Primary myelofibrosis-MSCs exhibit an increased *in vitro* and *in vivo* osteogenic potential

We further compared the abilities of MSCs from primary myelofibrosis patients ($n = 7$), essential thrombocytopenia/polycythemia vera patients ($n = 6$) and HDs ($n = 3$; P3-P5) to differentiate towards the osteogenic, chondrogenic, and adipogenic lineages. Fig. 3A shows that under specific conditions, MSCs from patients and from HDs similarly differentiated into cells with morphologic and cytochemical characteristics of adipocytes and chondrocytes, as shown after Oil-Red O or Alcian Blue staining. In contrast, under osteogenic differentiation conditions, MSCs from primary myelofibrosis patients showed an increased

capacity to mineralize compared with essential thrombocytopenias/polycythemia veras and HDs (attested by Von Kossa and Red Alizarin stainings) and in alkaline phosphatase activity (Fig. 3A). Red Alizarin quantification showed a significant increased mineralization at day 21 in primary myelofibrosis-MSCs compared with essential thrombocytopenia/polycythemia veras and HDs (159.8 ± 35.9 vs. 62.35 ± 17.7 and 50.9 ± 13.1 mg/L, $n = 3, 6$ and 7 , respectively, $P = 0.05$; Fig. 3A).

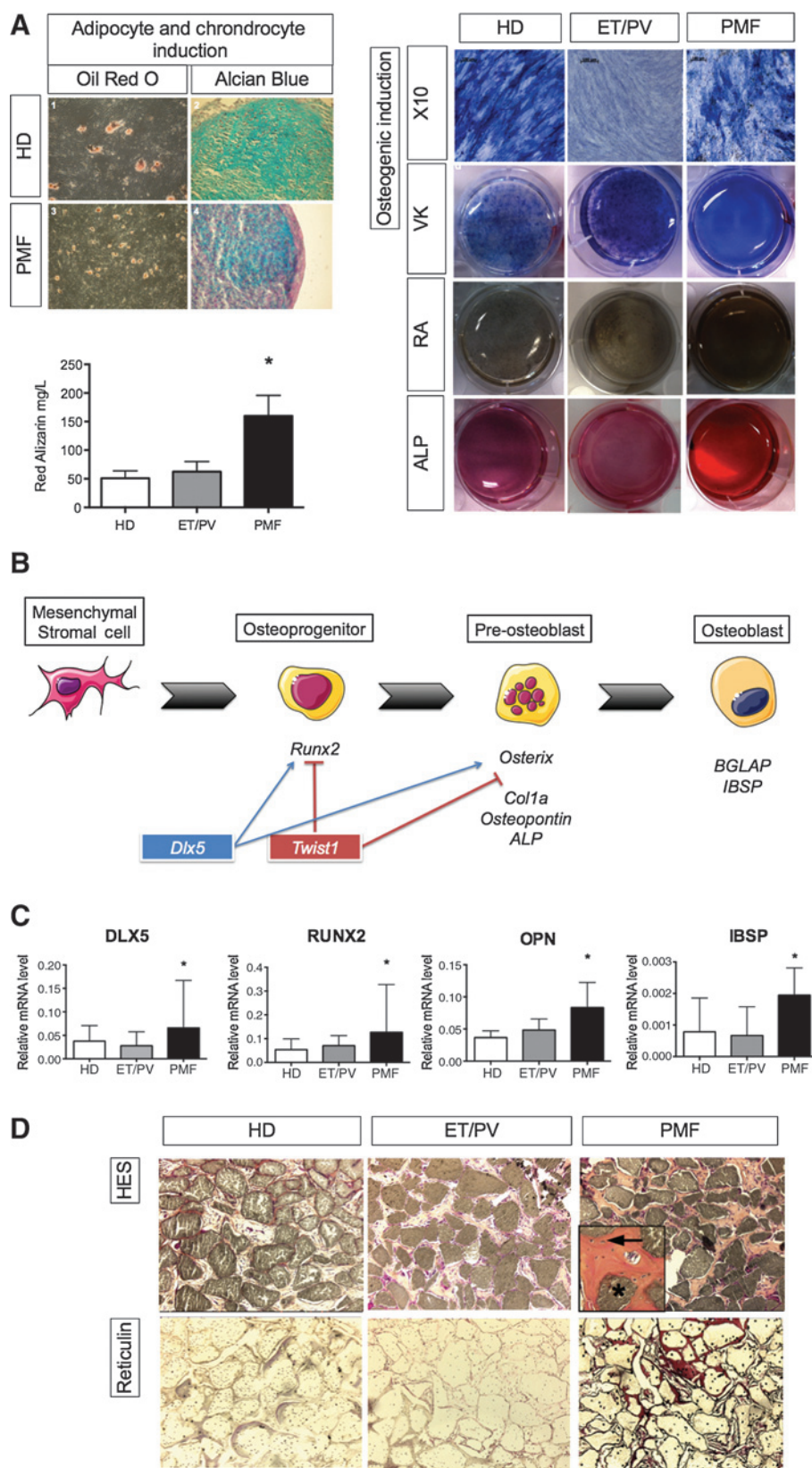
We further quantified the expression of genes involved in osteogenic differentiation. Figures 3B and C show that after 21 days in osteogenic conditions, the expression of *Runx2*, *Dlx5*, *osteopontin*, and *integrin-binding Sialo-protein (IBSP)* were significantly upregulated in differentiated MSCs from primary myelofibrosis compared with essential thrombocytopenia/polycythemia veras and HDs.

To confirm the increased osteogenic capability of primary myelofibrosis-MSCs, we investigated their *in vivo* osteogenic capacity by ectopic implantation into mice. Ten weeks postimplantation, mineralized bone tissue was observed in all implants and primary myelofibrosis-MSCs exhibited an increased osteogenic potential compared with HDs (Fig. 3D, $n = 3$ /primary myelofibrosis and HD, $n = 6$ /essential thrombocytopenia/polycythemia vera). Interestingly, the increased *in vivo* osteogenic capability was associated with an increased deposition of reticulin fibers, a mark of fibrosis. Fibrosis density was significantly higher after primary myelofibrosis-MSC implantation compared with essential thrombocytopenia/polycythemia veras and HDs (2.3 ± 1.6 vs. 0.9 ± 0.7 and 0.3 ± 0.2 AU, $P < 0.01$). Altogether, our results evidence that implantation of primary myelofibrosis MSCs, and not of essential thrombocytopenia/polycythemia vera MSCs, lead to fibrosis and increased ossification in murine ectopic ossicles similarly to that is observed in primary myelofibrosis bone marrow.

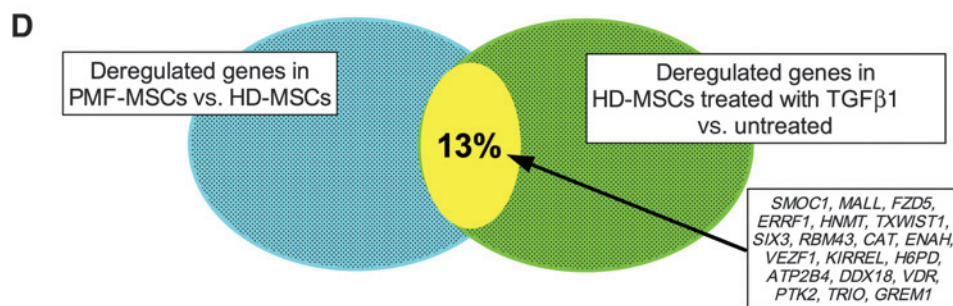
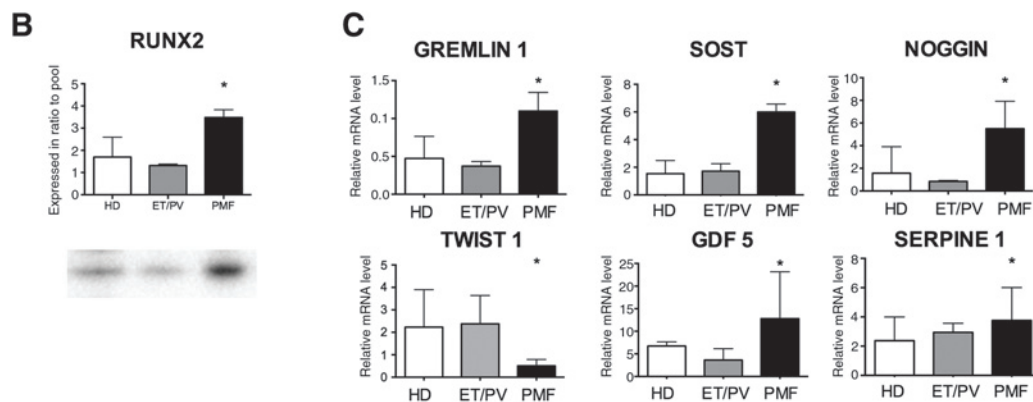
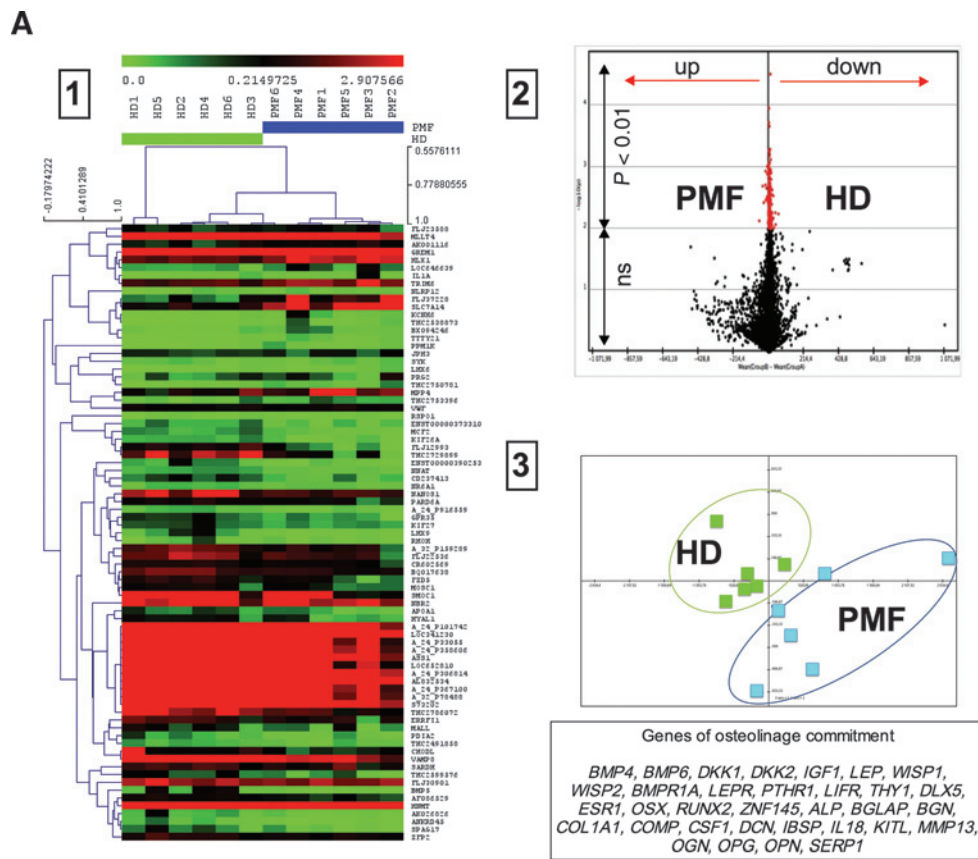
Primary myelofibrosis-MSCs show an original transcriptome signature related to osteogenic lineage and TGF β 1 signaling

To confirm alteration of primary myelofibrosis-MSC differentiation, we compared gene expression of MSCs from primary myelofibrosis patients ($n = 6$) and HD ($n = 6$; P2-P4). Transcriptome analysis with 24,000 targets revealed 173 significantly deregulated genes. Among them, 57 were downregulated more than 2-fold and 22 were upregulated more than 2-fold (Fig. 4A1 and A2; GSE44426). When focused on genes involved in lineage commitment (22), we observed that 9 of 33 genes regulating osteogenic commitment, including master genes such as *Runx2* and *Dlx5*, were significantly deregulated. Principal component analysis of these genes revealed a good clustering of primary myelofibrosis-MSCs compared with HDs arguing for a specific mRNA signature of primary myelofibrosis-MSCs (Fig. 4A3). Osteogenic lineage based clustering prompted us to investigate an osteoprogenitor signature in primary myelofibrosis-MSCs. We performed a bioinformatics assay by comparing published transcriptome of MSCs that were differentiated in osteoprogenitors and osteoblasts (23) with primary myelofibrosis-MSC transcriptome. This analysis revealed a gene expression pattern in primary myelofibrosis-MSCs consistent with a more advanced stage of differentiation toward osteoblast. *Runx2*, *Dlx5*, and *Msx1* are genes traditionally associated with osteoprogenitor state. We fail to evidence a significant increased expression of these genes, due to heterogeneity among patients (data not shown). However, *Runx2* protein was increased in primary myelofibrosis-MSCs

Figure 3. Primary myelofibrosis (PMF)-MSCs exhibit an increased *in vitro* and *in vivo* osteogenic potential. A, MSCs from healthy donors (HD, $n = 3$) and from primary myelofibrosis patients ($n = 6$) were cultured in adipogenic medium (Oil-Red-O staining; magnification, $\times 10$) or chondrogenic medium (Alcian Blue staining; magnification, $\times 20$) for 21 days (A, left). Osteogenic differentiation of MSCs from primary myelofibrosis, essential thrombocytopenia (ET)/polycythemia vera (PV) patients or healthy donors were performed during 21 days: alkaline phosphatase (ALP) staining ($\times 10$), Von Kossa, red alizarin, and alkaline phosphatase. Pictures representative of six experiments, MSCs at passage 6 (A, middle). Red alizarin quantification was performed [HD, $n = 3$; essential thrombocytopenia (ET)/polycythemia vera (PV), $n = 6$; primary myelofibrosis, $n = 7$]; results are expressed in mg/mL as mean \pm SD; *, $P < 0.05$, (A, left bottom). B, ontogeny of osteoblast and regulatory control of osteoblast lineage from MSCs to osteoblast. Blue, positive regulators; red, inhibitor factor; black, genes involved at different stage of differentiation. C, gene expression of *DLX5*, *Runx2*, *osteopontin* (*OPN*), and *IBSP* tested after 21 days in osteogenic condition. Results are normalized on housekeeping genes expression (*β -actin*, *GAPDH*, *RPL38*, *RPL13a*, and *18s*). $n = 6$; *, $P < 0.05$. D, MSCs from HD ($n = 3$), essential thrombocytopenia (ET)/polycythemia vera (PV; $n = 6$), and primary myelofibrosis patients ($n = 3$) mixed with calcium phosphate particles were subcutaneously implanted in nude mice on scaffolds and examined 10 weeks later; implants were retrieved, embedded, and sections analyzed after HES and reticulin staining (magnification, $\times 10$). Inset, HES and reticulin staining of primary myelofibrosis-MSCs represent focus on osteocytes (arrow) embedded in new bone formed in the particle (black star) interspaces.



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compared with essential thrombocytopenia/polycythemia vera and HDs, as assessed by Western blot analysis (Fig. 4B). We also focused on bone master gene regulators and showed upregulation of *Gremlin1*, *Noggin*, *GDF5*, *Sclerostin*, and downregulation of *Twist1* in primary myelofibrosis patients compared with essential thrombocytopenia/polycythemia vera and HDs (Fig. 4C). Altogether these data indicate a specific shift of differentiation of primary myelofibrosis-MSCs toward osteogenic lineage.

Finally, the upregulation of *Serpine1*, a well-known target of TGF β 1 (24), in association with the pivotal role of TGF β 1 in primary myelofibrosis, prompted us to compare primary myelofibrosis-MSC transcriptome with the signature of HD TGF β 1 treated bone marrow-MSCs (GSE46019; ref. 25). Figure 4D shows that 13% of significantly deregulated genes in primary myelofibrosis-MSCs were common with genes significantly deregulated in TGF β 1-treated HD-MSCs. Altogether these data suggested a TGF β 1 signature in primary myelofibrosis-MSC transcriptome deregulation.

TGF β 1 is involved in the osteogenic deregulation of primary myelofibrosis-MSCs

We previously showed that primary myelofibrosis-MSCs overproduced TGF β 1 and BMP-2 and that their osteogenic abilities were increased compared with that of HD-MSCs. Therefore, taking into account the stimulation effect of TGF β 1 on osteogenic commitment, we compared the effects of a TGF β 1 treatment (10 ng/mL for 48 hours) on MSCs isolated from HDs and primary myelofibrosis patients. We first confirmed TGF β RI and RII expression by primary myelofibrosis and HD MSCs (data not shown). Western blot analysis showed a significant higher phospho-Smad2 level in primary myelofibrosis-MSCs at steady state, without addition of TGF β 1, suggesting that these cells are constantly TGF β 1-stimulated (Fig. 5A, $n = 5$). Under TGF β 1 stimulation, we observed an increase of phospho-Smad2 levels in HDs and primary myelofibrosis patients. However, fold change in pSmad2 level after stimulation by TGF β 1 was not significantly different between HD and patients (3.4 ± 1.6 vs. 4.2 ± 1.3 , respectively, $n = 5$, $P = 0.15$). RQ-PCR analysis focused on *Runx2*, *Dlx5*, *Osterix*, and *Twist1* revealed a confident clustering by principal component analysis (Fig. 5B), demonstrating a specific regulation of these genes in primary myelofibrosis-MSCs in response to TGF β 1. We then hypothesized that endogenous TGF β 1 could be involved in primary myelofibrosis osteogenic impairment. To test this hypothesis, we inhibited TGF β 1 type I receptor ALK5 by a SB-431542 inhibitor treatment during the *in vitro* osteogenic differentiation of primary myelofibrosis-MSCs. In

presence of SB-431542, Von Kossa and Red Alizarin stainings as well as alkaline phosphatase activity showed an increased mineralization in MSC cultures in both patients and HDs. Red Alizarin quantification confirmed the increased mineralization after 21 days of culture in the presence of SB-431542 (primary myelofibrosis: $1,485 \pm 493$ vs. 519 ± 313 mg/mL, $P < 0.05$, $n = 7$; HDs: $1,840 \pm 191$ vs. 51 ± 13 mg/mL, $P < 0.05$); this increase was 3.5 higher in HDs (32 ± 15) than in primary myelofibrosis (9 ± 7 ; $P < 0.05$; Fig. 5C), suggesting a different response of MSC osteogenic differentiation to endogenous TGF β 1 in patients compared with HDs. As BMP-2 was also overexpressed by primary myelofibrosis-MSCs, we tested the inhibition of BMP-2 by LND-193189 during osteogenic differentiation. In contrast and surprisingly, HDs and PMF-MSCs exhibited a similar increased mineralization in the presence of LND-193189, with nonsignificant difference between their respective fold changes (26 ± 7 in HDs vs. 21 ± 7 in primary myelofibrosis). Similar behavior trend in primary myelofibrosis and HDs with LND-193189 suggests that BMP-2 is not involved in the osteogenic impairment. Taken together, our results reveal a specific response of primary myelofibrosis-MSCs to endogenous TGF β 1 and suggest its involvement in their osteogenic impairment.

Discussion

Although many somatic mutations have been discovered in the clonal HSCs, from the most commonly known *JAK2* to the most recent *calreticulin* (10), the pathogenic relevance of these mutations in primary myelofibrosis pathogenesis is currently unclear. Up to now, bone marrow microenvironment alterations were considered as a reactive counterpart of the cytokine production by clonal hematopoietic cells (26). Recently, more and more evidences argue for a relevance of the "bad seed in bad soil" concept in which the bad soil (altered stroma) endorsed the bad seed (clonal HSCs) development in primary myelofibrosis (2). However, evidences for acquired alterations of stromal cells are lacking in patients.

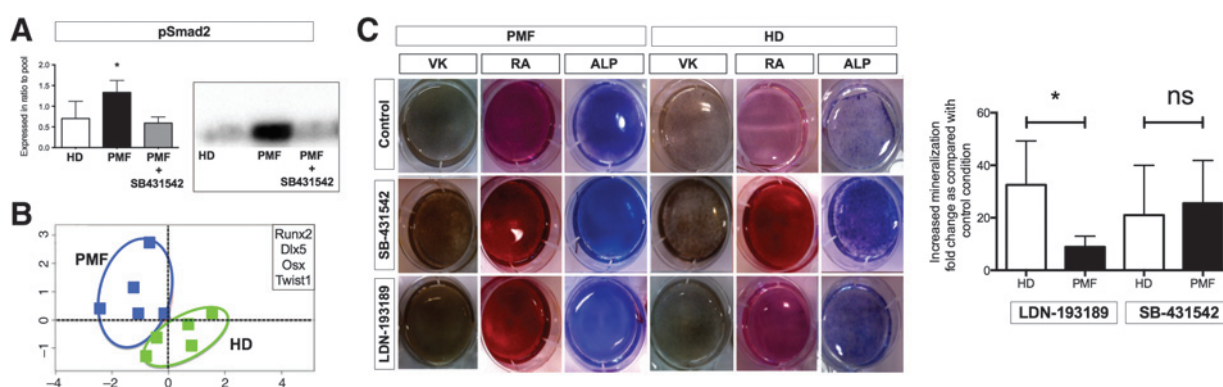
In the current study, we identified a molecular and functional signature of primary myelofibrosis-MSCs that is characterized by an increased endogenous production of TGF β 1, BMP-2, and GAGs and by an abnormal osteogenic potential that persists *in vitro*, that is, in the absence of any stimulation by hematopoietic cells and that is dependent on TGF β 1 pathway activation. These results are not observed with MSCs from essential thrombocytopenia and polycythemia vera patients.

As reported in other hematologic malignancies, medullar MSCs of primary myelofibrosis patients do not evidence differences

Figure 4.

Primary myelofibrosis (PMF)-MSCs show an original transcriptome signature related to osteogenic lineage and TGF β 1. A1, transcriptome analysis from primary myelofibrosis patients (PMF 1-6, $n = 6$) compared with healthy controls (HD 1-6, $n = 6$). Genes differentially expressed revealed a specific clustering of patients versus controls. A2, volcano plot shows significantly deregulated genes (red, $n = 173$, $P < 0.01$) and nonsignificantly expressed genes (black); number of more than 2-fold downregulated genes ($n = 57$) in primary myelofibrosis-MSCs is higher than 2-fold upregulated ($n = 22$). A3, principal component analysis of significantly deregulated genes involved in osteogenic commitment revealed a good clustering of MSCs isolated from patients (PMF) and from controls, principal component 1 represents 84% of clustering whereas principal component 2 represents 15%. ns, nonsignificant. B, expression of protein Runx2 in MSCs from primary myelofibrosis (black bar), essential thrombocytopenia (ET)/polycythemia vera (PV) patients (gray bars, $n = 6$), and HDs (white bar) cultivated up to 48 hours in α -MEM medium supplemented by FBS 10%. Results are expressed as a ratio to the median expression of the targeted proteins in all samples of the same experiment; blots are representative of experiments ($n = 5$ in each group; *, $P < 0.05$). C, RQ-PCR analysis of gene expression level in MSCs from patients (black bars, $n = 6$), essential thrombocytopenia (ET)/polycythemia vera (PV) patients (gray bars, $n = 6$), and HDs (white bars, $n = 6$); results are normalized on housekeeping genes expression (*β -actin*, *GAPDH*, *RPL38*, *RPL13a*, and *18s*); *, $P < 0.05$. D, Venn diagrams of genes deregulated in primary myelofibrosis-MSCs compared with HD and HD-MSCs cultured in TGF β 1 medium up to 48 hours revealed that more than 10% of significantly deregulated genes in primary myelofibrosis transcriptome are common with genes involved in TGF β challenging of MSCs.

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**Figure 5.**

TGF β 1 is involved in the osteogenic deregulation of primary myelofibrosis (PMF)-MSCs. A, expression of protein pSmad2 in MSCs from primary myelofibrosis (black bar) or HD (white bar) cultivated up to 48 hours in α -MEM medium supplemented by FBS 10% and from primary myelofibrosis cultivated in the same conditions in addition to SB-431542 (10 μ mol/L; gray bar). Results are expressed as a ratio to the median expression of the targeted proteins in all samples of the same experiment; blots are representative of experiments ($n = 5$ in each group; *, $P < 0.05$). B, principal component analysis of gene expression (DLX5, Runx2, TWIST1, Osterix) under TGF β 1 stimulation (10 ng/mL during 48 hours). Blue circle clusters primary myelofibrosis-MSCs; green circle clusters HD MSCs ($n = 5$ in each group). C, MSCs from primary myelofibrosis patients (PMF) or controls were cultured up to 21 days in osteogenic medium with or without SB-431542 (10 μ mol/L) or LDN-193189 (1 μ mol/L). Von Kossa (VK), Red Alizarin (RA), and alkaline phosphatase (ALP) staining were performed. Primary myelofibrosis-MSCs showed increased mineralization (VK and RA) and ALP activity during osteogenic differentiation. TGF β 1 inhibition is associated with an increased osteogenic differentiation of control-MSCs and primary myelofibrosis-MSCs, attested by a higher VK/RA and ALP staining. Inhibition of BMPs leads to an increase osteogenic ability in primary myelofibrosis as well as in HD MSCs. Pictures are representative of 6 experiments (left). Quantification of Red Alizarin staining showed that primary myelofibrosis-MSCs ($n = 7$) underwent a significant lower increase of mineralization compared with controls ($n = 3$) during osteogenic differentiation with TGF β 1 inhibition, but the same ratio of increased mineralization when BMPs were inhibited (*, $P < 0.05$; right). ns, nonsignificant.

with HD-MSCs regarding their proliferation, phenotype, and hematopoiesis supporting abilities (8, 27). Primary myelofibrosis-MSCs neither demonstrate senescence features, contrasting with data from Avanzini and colleagues (8) in which cytoreductive therapy might have interfered with the senescence process.

Whereas primary myelofibrosis-MSCs showed similar adipogenic and chondrogenic differentiation capabilities than HD-MSCs, our data clearly argue for an impairment of their osteogenic abilities that fits with the osteosclerosis described in patients (1) as shown by their increased capacity to mineralize extracellular bone matrix *in vitro* and to form new bone *in vivo* in immunodeficient mice. Even if primary myelofibrosis shares common features with essential thrombocytopenia and polycythemia vera, our results argue for a specific impairment of MSCs osteogenic abilities in primary myelofibrosis patients. Such osteogenic impairment is associated with a deregulated expression of main actors of bone formation such as *Runx2*, *Dlx5*, and *Osterix* and of their regulators *Twist1*, *Noggin*, *GDF5*, and *Gremlin*. Whereas *Noggin* is overexpressed in primary myelofibrosis-CD34⁺ cells (28) and is known to interact with GAGs (29), the reason why primary myelofibrosis-MSCs express high levels of *Noggin* and *Gremlin*, two BMP-2 antagonists, remains to be elucidated. Interestingly, as observed in primary myelofibrosis-MSCs, *Twist1* downregulation and *GDF5* overexpression are respectively associated with osteogenic enhancement and osteoprogenitor state (30, 31), suggesting a pro-osteogenic commitment of primary myelofibrosis-MSCs.

Regulation of osteogenic differentiation involved several cytokines including TGF β 1 and BMP-2. Both cytokines are produced in excess by primary myelofibrosis-MSCs as confirmed by the absence of contaminating CD45⁺ hematopoietic cells in MSC cultures. TGF β 1, a leading cytokine in primary myelofibrosis, is reported to be released by megakaryocytes and monocytes (32) from patients; its overproduction and activation in primary mye-

lofibrosis-MSCs is reported for the first time in this study. Among proteins involved in TGF β 1 regulation, GAGs such as HS and CS play an original role by modifying local concentrations of HBP, leading to a gradient of their concentration (33). GAGs protect and trigger the activity of HBP and optimize binding and activity of growth factors such as TGF β 1 and BMP-2 (33). In primary myelofibrosis, we demonstrated an increased production of GAGs *in vitro* by MSCs and *in vivo* on bone marrow biopsies; this overproduction is higher in evolved patients (fibrosis grade 3). It is reported that enhancement of GAGs promotes osteogenic commitment (34) and that TGF β 1 exerts a dual action on osteoblast differentiation. Actually, whereas TGF β 1 disruption in mice results in bone defects, its overexpression displays an osteoporosis-like phenotype (35), suggesting that TGF β 1 enhances the proliferation and early differentiation of osteoblasts, but impairs their terminal differentiation. In contrast to TGF β 1, relation of GAGs with BMP-2 is still unclear as HS is reported either to enhance (36) or to disrupt (37) BMP-2 signaling.

TGF β 1 and BMP-2 overexpression in primary myelofibrosis-MSCs prompted us to study the potential involvement of both cytokines in the pro-osteogenic commitment of primary myelofibrosis-MSCs. Primary myelofibrosis-MSCs exhibit an increased level of phospho-Smad2 demonstrating that TGF β pathway is already highly activated in these cells compared with HD-MSCs. This is consistent with increased TGF β 1 expression in primary myelofibrosis-MSCs, suggesting that primary myelofibrosis-MSCs are constantly primed by endogenous TGF β 1 to an osteoprogenitor state. Such commitment will lead to the increase ability of osteogenic differentiation of primary myelofibrosis-MSCs when cultured in osteogenic conditions (Fig. 6, adapted from ref. 23). In accordance, *Serpine1*, which is expressed early during osteogenic differentiation, and upregulated by TGF β 1 (38), is overexpressed in primary myelofibrosis-MSCs arguing for their TGF β 1-induced preosteogenic state. As expected, when TGF β 1 pathway was

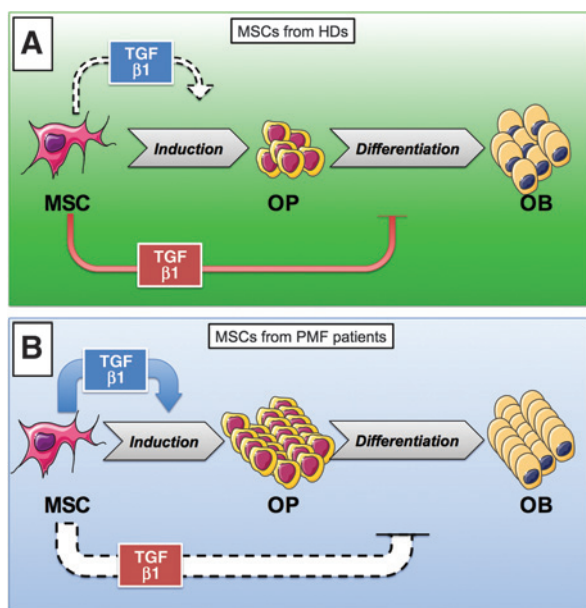


Figure 6.

TGFβ1 involvement in osteogenic impairment of primary myelofibrosis (PMF)-MSCs. A, in nonpathological healthy donor MSCs, osteogenic differentiation features two opposite effects of TGFβ1; a minor effect (arrow with dashed-line) is represented by the recruitment of osteoprogenitors (OP; cells with pink nuclear) from MSCs and the main effect (arrow with full-line) by an inhibition of late stages of osteoblastic differentiation (OB; cells with blue nuclear). B, in primary myelofibrosis-MSCs, self-production of TGFβ1 is increased compared with HDs and leads to an increased pool of osteoprogenitor-like cells, and then, under osteogenic induction to an increased mineralization and alkaline phosphatase activity.

inhibited by addition of a TGFβ1 R1 inhibitor, the osteogenic capability of MSCs from patients and from HDs are both increased. However, the relative increase in mineralization is less important for primary myelofibrosis-MSCs likely due to their higher steady-state osteogenic commitment. Altogether, our data suggest that TGFβ1 participates in the higher osteogenic ability of primary myelofibrosis-MSCs.

In contrast, inhibition of BMP pathway by LDN-193189, an inhibitor of BMP receptors ALK2, 3 and 6, had an unexpected stimulatory effect on osteogenic differentiation in MSCs from both primary myelofibrosis patients and HDs. This was quite unexpected as BMP-2 is a major osteogenic enhancer and as its inhibition should have led to a decreased rather than an increased MSC osteogenic differentiation. The reason of such discrepancy is still unknown but it might be explained by the fact that LDN-193189 is also able to inhibit other BMPs than BMP-2 and among them, some inhibitors of BMPs (20).

The pathophysiologic mechanisms leading to the TGFβ1 overproduction by primary myelofibrosis-MSCs need to be studied. To date, some chromosomal abnormalities have been described in primary myelofibrosis-MSCs (8), but no recurrent mutations potentially responsible for TGFβ1 overproduction have been identified. TGFβ1 is secreted as latent TGFβ1 and its activation is tightly regulated by cell traction forces and proteinases (39). In primary myelofibrosis, the increased reticulin production by primary myelofibrosis-MSCs that would modify the stiffness of the bone marrow stroma could participate in this activation process.

Epigenetic modifications could be involved in the maintenance of this phenotype. The deregulated expression of miRNAs such as miR-34 and miR-106 (our unpublished data), known to activate TGFβ1 signaling pathway (40) and in relation with inflammaging (41), are in agreement with our hypothesis. The high inflammatory context in which primary myelofibrosis is developing could therefore promote epigenetic alterations of primary myelofibrosis patient's stroma, resulting in MSC imprinting.

While demonstrating intrinsic functional alterations of medullar MSCs from primary myelofibrosis patients, our results do not preclude that, *in vivo*, stromal cells are targets of the cytokine storm generated by the pathologic hematopoietic cells (2). The issue of whether the MSC abnormalities evidenced in the present work could have been *in vivo* triggered by the hematopoietic clone during the course of the disease remains to be addressed. As elegantly proposed by H.C. Hasselbalch (42), chronic inflammation may be a driver of clonal evolution in patients with MPNs, from early disease state (essential thrombocytopenia and polycythemia vera) to more progressive disease state (primary myelofibrosis). The fact that osteogenic alterations are not observed in essential thrombocytopenia and polycythemia vera is in accordance with this assumption. Thus, in primary myelofibrosis patients and over decades, it could be suggested that the stroma is inflammatory-imprinted by clonal hematopoietic cells to an "autonomous" state where it becomes independent of hematopoietic cell stimulation, rendering this inflammatory vicious circle unbreakable without combined stroma-targeted therapies.

Our data indicate that primary myelofibrosis-MSCs display an "intrinsic" osteogenic signature and that their increase differentiation into osteoblasts is partly dependent of endogenous TGFβ1 production and activation. These alterations, maintained over several *in vitro* passages and therefore independent from any local stimulation by hematopoietic cells, raise the issue of the reversal of fibrosis after HSC transplantation reported in some patients. However, the purely reactive conception of bone marrow alterations is questioning and results are quite conflicting. Actually, some studies report that decrease of fibrosis is slow, incomplete (43, 44), and inconstant (45) while others show that dose-reduced conditioning, followed by allogeneic stem cell transplantation, resulted in a rapid resolution of bone marrow fibrosis (46). Intriguingly, decrease of fibrosis is not correlated with megakaryocytes that are the main source of profibrotic cytokines, suggesting that replacement of clonal hematopoiesis is not the only reason responsible for fibrosis regression (44). Regarding osteosclerosis, data are more homogenous: no improvement is observed after transplantation (44, 47). Altogether, these results strengthen our hypothesis that curing the leukemic cells is not sufficient to cure the stromal alterations.

In conclusion, our results are of pathophysiologic importance as they evidence intrinsic functional alterations of mesenchymal stromal cells and of their derivatives in primary myelofibrosis. The increased osteogenic potential of primary myelofibrosis-MSCs associated with increased fibrosis contrasts with data from Avanzini and colleagues reporting a decreased osteogenic ability in MPN-MSCs (8) but fits with the osteomyelosclerosis observed in patients. Besides their role in osteogenesis, TGFβ1 and BMPs are involved in regulation of HSC dormancy and therefore, they may contribute to leukemic stem cell maintenance as recently reported in another myeloproliferative neoplasm such as chronic myeloid leukemia (48). Taking into account the cross-influence between the bone marrow extracellular matrix composition and the

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proliferation/differentiation capability of hematopoietic stem cells (5), the stimulating issue concerning the impact of stromal cell alterations on hematopoiesis needs to be elucidated.

By being "bad stromal cells," MSCs take entirely part in the "bad seed in bad soil" concept (1) and strengthen the importance of stromal cells in the development of a neoplasia. Therefore, MSCs from patients are good candidates for niche-targeted therapies that, in association with drugs eradicating the hematopoietic clone, would improve patient treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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