INTRODUCTION

Philadelphia-negative chronic myeloproliferative neoplasms (MPNs) are a heterogeneous group of related clonal malignant hemopathies comprising of polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). MPNs arise from the transformation of hematopoietic stem cells (HSC) and in the majority of cases caused by JAK-STAT (Janus kinase/signal transducers and activators of transcription) pathway activating mutations in the JAK, MPL (myeloproliferative leukaemia virus), and calreticulin gene, respectively.1

Clinically, PV and ET are characterized by the overproduction of one or more mature myeloid blood cell lineages, whereas the
predominant feature of PMF is BM fibrosis causing extramedullary hematopoiesis, splenomegaly, and anemia.

Current treatment options, except for allogeneic stem cell transplantation, are not curative and aim at complication prevention, symptom improvement, and risk minimization of progression to fibrosis or leukemia. The JAK1/2 inhibitor Ruxolitinib (Ruxo) is the first approved drug for the treatment of intermediate/high-risk MF and PV. Although designed as a specific targeted therapy, Ruxo does not cure the disease but has nevertheless demonstrated impressive clinical effectiveness including normalization of blood counts, reduction in spleen size, and relief of MPN-related symptoms. Importantly, Ruxo treatment has been reported to prolong survival in MF and to improve or even reverse BM fibrosis after long-term treatment.

The effects of Ruxolitinib on blood counts and spleen size can be directly attributed to the drug-induced inhibition of dysregulated JAK-STAT signaling in mutated hematopoietic cells. However, normalization of the inflammatory stromal environment in MPN and improvement of BM might be—at least in part—due to Ruxo effects on other cell types. Of note, Ruxolitinib is effective also in JAK2 non-mutated patients and has been demonstrated to affect dendritic cells, NK cells, and T cells. Whether non-hematopoietic BM stromal cells (MSC), which are not only important elements of the BM stroma and critical regulators of hematopoiesis, but also contribute to the proinflammatory environment and BM fibrosis, are possible targets of Ruxo has not yet been investigated. Of note, MSC from MPN patients have been demonstrated to not harbor MPN-specific mutations of the JAK-STAT pathway.

This study therefore aimed to investigate the in vitro effects of Ruxolitinib on healthy donor MSC and MSC from JAK2 V617F+ MPN patients. The data clearly demonstrated that Ruxo inhibited JAK-STAT signaling in MSC. However, this did not translate into a clear inhibition of MSC proliferation and clonogenicity, but caused marked changes in the MSC cytokine profile. These data thus indicate that JAK2 inhibition might affect paracrine stromal contributions in the inflammatory MPN niche in vivo, which adds new perspectives to future therapeutic approaches targeting the stromal compartment in hematological diseases.

### 2 Patients and Methods

#### 2.1 Healthy and patient bone marrow donors

Bone marrow aspirates were harvested from the iliac crest of consenting healthy donors (n = 10) and patients (n = 7) with myeloproliferative neoplasms (MPNs) and known JAK2 mutational status (see Table 1). The study and all related protocols were performed according to the guidelines of the Regional Ethical Review Board in Lund (Dnr 2009/532 and Dnr 2014/776) and in accordance with the 2013 revised Helsinki Declaration.

#### 2.2 Bone marrow mononuclear cells isolation and MSC culture

Bone marrow mononuclear cells were isolated as described previously by density gradient centrifugation (LSM 1077 Lymphocyte; PAA) after incubation with RosetteSep Human MSC Enrichment Cocktail (Stem Cell Technologies) for lineage depletion (CD3, CD14, CD19, CD38, CD66b, glycophorin A). PharmLyse was used for additional red blood cell removal. Cells were enumerated using a hemocytometer, and they were used either fresh or as third- or fourth-passage MSC after culture in expansion media (StemMACS MSC Expansion Media, Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 1% antibiotic–antimycotic solution (ThermoFischer Scientific, Sweden).

#### 2.3 Preparation and administration of JAK1/2 inhibitor Ruxolitinib

Ruxolitinib (INCB018424) was purchased from Selleckchem (S1378, Rungsted, Denmark). The compound was dissolved in 100% DMSO and used at the following final concentrations: 0.2 μmol/L, 0.5 μmol/L, 1 μmol/L, 5 μmol/L, and 10 μmol/L (the final maximum DMSO concentration in culture did not exceed 0.06%). Working solutions of Ruxolitinib and corresponding DMSO-containing culture media were fully replaced every 48 hours.

### TABLE 1 Patient bone marrow donor characteristics and diagnosis

| Patient code | Age | Gender | Diagnosis | JAK2 mutation | Treatment at time of BM aspiration |
|--------------|-----|--------|-----------|---------------|-----------------------------------|
| P-MSC1       | 56  | Female | MPN-U     | V617F         | PegInterferon alpha 2a            |
| P-MSC2       | 73  | Female | ET        | V617F         | None                              |
| P-MSC3       | 66  | Female | PV        | V617F         | None                              |
| P-MSC4       | 64  | Male   | PV        | V617F         | Phlebotomy (2 x before diagnostic BM) |
| P-MSC5       | 67  | Male   | PV        | V617F         | None                              |
| P-MSC6       | 73  | Male   | ET        | V617F         | Hydroxyurea                       |
| P-MSC7       | 84  | Male   | MF        | V617F         | None                              |

BM, bone marrow; ET, Essential Thrombocytopenia; MF, Myelofibrosis; MPN, myeloproliferative neoplasm; MPN-U, myeloproliferative neoplasm, unclassified; PV, Polycythemia Vera.
2.4 | Cytotoxicity assays

For short-term and long-term cytotoxicity assays, 2500 MSCs/well were added to a 96-well plate in 100 μL of medium (3-5 replicates/condition). The plate was incubated overnight in a humidified incubator (at 37°C, 5% CO₂) to allow for MSC adherence, followed by complete media replacement with either MSC medium (control), only DMSO-containing media (DMSO controls) or media containing Ruxolitinib at different concentrations. The Cell Counting Kit-8 (CCK8, Merck, Darmstadt, Germany) assay was used for cell counting in the short-term (48 hours) exposure experiments according to the manufacturer’s instructions. At indicated time-points, 10 μL of CCK8 were added to each well and the plates were incubated for 3 hours. The optical density was measured at 450 nm with 620 nm as reference using a microplate reader. In the long-term cytotoxicity assays live and dead cells were enumerated using a hemocytometer and Trypan Blue staining (Trypan Blue Solution, 0.4%; Fischer Scientific, Sweden).

2.5 | Clonogenicity assay

Colony formation was evaluated using freshly isolated BM-MNC from 9 different healthy BM donors. CFU-F cultures were performed in 6-well plates as previously described.20 Briefly, 20,000 MNC/cm² were seeded in triplicates per group. Full media changes with Ruxolitinib-containing medium and medium with corresponding doses of DMSO, respectively, were performed after 72 hours and every 48 hours thereafter. Fibroblastic colonies (≥40 cells) were counted microscopically after 14 days following staining with 1% Crystal Violet. Colony diameters (mm) from 5 independent CFU-F experiments (3 wells per group) were measured using gridded cell culture plates.

2.6 | cDNA synthesis and qPCR

RNA was extracted from passage 2 and 3 MSC from patients with JAK2V617F MPNs using the RNeasy Mini kit (Qiagen, Sollentuna, Sweden) according to the manufacturer’s instructions. cDNA was synthesized employing the Transcriptor first strand cDNA synthesis kit (Roche, Stockholm, Sweden) on a C1000TM Thermal Cycler (Bio-Rad, Hercules, CA, USA) followed by real-time PCR using Fast SYBR master mix (Applied Biosystems, Stockholm, Sweden) according to the manufacturer’s instructions. The primer sequences used are listed in Table S1. Human GAPDH was used as a reference gene, and the relative expression of each mRNA was determined using the relative −ΔΔCt method.

2.7 | IL-6 stimulation of healthy donor MSC

Human recombinant IL-6 (Cell Signaling Technology, BioNordika, Stockholm, Sweden) was diluted in sterile phosphate-buffered saline (PBS, Hyclone, GE Healthcare Life Sciences, Uppsala, Sweden) with 5% FBS and added to the cultured MSC at a final concentration of 100 ng/mL.

2.8 | Western blot analysis

Protein lysates were extracted from passage 3 (P3) cultured MSC as follows. Briefly, cell cultures were washed 3 times with ice-cold TBS (Tris Buffered Saline 10x, Fisher Scientific, Gothenburg, Sweden) followed by addition of cold lysis buffer Nonidet P40 (ThermoScientific, Stockholm, Sweden) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride (PMF), 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail 3 (all from Sigma-Aldrich, Stockholm, Sweden). Cell lysates were transferred into Eppendorf tubes and centrifuged at 15871 x g for 2.5 minutes at 4°C. The supernatants were collected, protein contents were measured using the Pierce BCA Protein Assay Kit (ThermoScientific, Stockholm, Sweden), and supernatants were stored at −80°C.

Protein electrophoresis was performed using 7.5% and 10% precast gels (Mini-PROTEAN TGX™, Bio-Rad, Solna, Sweden), and the protein bands were transferred overnight on ECL nitrocellulose membranes (Amersham Hybond, GE Healthcare Life Sciences, Uppsala, Sweden). The membranes were blocked with Superblock/TBS/Tween20 for phospho-proteins and Superblock/PBS/Tween-20 for whole proteins, respectively (both from ThermoFisher Scientific, Gothenburg, Sweden) for 1 hour at room temperature and then incubated with primary antibodies overnight. Antibodies used for Western Blot analysis are listed in Table S2. After washing and incubation with the secondary antibody membranes were visualized with Amersham ECL Advance Western Blotting Detection kit (GE Healthcare Life Sciences, Uppsala, Sweden) on a Bio-Rad imaging system.

2.9 | Cytokine assays

The cytokine array from R&D systems (Proteome Profiler™ Array, Human Cytokine Array Panel A, Biotechnie, Abingdon, UK) was employed to assess the levels of 36 selected cytokines and chemokines in the culture supernatant of P3 MSC (unstimulated, IL-6-stimulated, and Ruxo-treated plus IL-6 stimulated, respectively). The assay was performed according to the manufacturer’s instructions. Briefly, MSC were serum starved in DMEM/F-12 (ThermoFisher Scientific, Gothenburg, Sweden) for 24 hours and hIL-6 (300 ng/mL) or diluent was added as indicated. After an additional 24 hours, cells were incubated with Ruxolitinib-containing medium (1 μmol/L) for another 36 hours. Culture supernatants were collected, centrifuged at 1000 x g for 15 minutes at 4°C, and stored at −80°C until being analyzed with the R&D cytokine array. Medium only or medium containing hIL-6 was used to control for background noise. Array chemiluminescent signals were captured on high sensitivity films (Amersham Hyperfilm ECL, GE Healthcare Life Sciences, Uppsala, Sweden). The arrays were scanned, and data were quantified as pixel density measurements using the Quantity One 1-D analysis software (v4.6.3, Bio-Rad Laboratories Inc., Sweden).

2.10 | ELISA

Human chemokine CCL2/MCP-1 and IL-6 cytokine levels were assessed in cell culture supernatants of P3 MSC from patients with
JAK2V617F MPNs and age-matched controls using Quantikine ELISA kits (R&D systems, Bioteche, Abingdon, UK) following the manufacturer’s instructions. First, MSC were serum starved in DMEM/F-12 for 24 hours followed by treatment with 5 μmol/L Ruxolitinib for 24 hours. Culture supernatants were collected, centrifuged at 1000 x g for 15 minutes at 4°C, and stored at −80°C in 1% FBS until analysis. Microplate readings were analyzed with SoftMax Pro 6.4 software (Molecular Devices, CA, USA).

2.11 | Bio-Plex cytokine assay

Cell culture supernatant samples from healthy donor MSC and JAK2V617F positive patient MSC (passage 3 and 4) were used to assess the levels of MMP-2 matrix metalloproteinase and TGF-β1 in single-plex assays (Bio-Rad, Solna, Sweden). Briefly, MSC from healthy controls and from MPN patients were serum starved in DMEM/F-12 for 24 hours and treated with 1 μmol/L Ruxolitinib for 36 hours thereafter. Culture supernatants were collected and centrifuged at 1000 x g for 15 minutes at 4°C to remove cell particles. The supernatants were stored in BSA 0.5% at −80°C until further use. All assays were performed according to the manufacturer’s instructions and run on a Bio-Plex 200 system.

2.12 | Statistical analysis

One-way or two-way analysis of variance (ANOVA) combined with Tukey’s multiple comparisons tests for inter-group comparisons was applied using the GraphPad Software, (v7.00, La Jolla California USA, www.graphpad.com). Statistical significance was set at *P ≤ .05.

3 | RESULTS

3.1 | Ruxolitinib only slightly affected healthy donor MSC survival and proliferation

First, the direct effects of Ruxolitinib on proliferation and survival of cultured healthy donor MSC were investigated. As shown in Figure 1A, increasing concentrations of Ruxolitinib (0.2 μmol/L, 0.5 μmol/L, 1 μmol/L, 5 μmol/L and 10 μmol/L) resulted in slightly increased numbers in the lower doses ranges and a tendency to reduced numbers of Ruxolitinib-treated MSC relative to their corresponding DMSO controls at higher dose levels in short-term exposure experiments (6 hours, 12 hours, 24 hours and 48 hours). However, differences in cell survival rates were not statistically significant. Nevertheless, time was identified as a significant factor for cell survival at 24 hours. In the next step, the long-term toxicity of Ruxolitinib on MSC was assessed (Figure 1B). Again, a decrease in cell numbers up to about 50%-60% was observed at the highest concentrations (5 μmol/L and 10 μmol/L, day 14 and 21); however, differences were not statistically significant when compared to corresponding DMSO controls neither with regard to time nor dose.

In comparison, proliferation of JAK2V617F-positive human erythroleukemia cells (HEL) was significantly affected by Ruxolitinib (Figure S1). As expected and in accordance with previous reports, HEL cell proliferation was inhibited in a dose-dependent fashion by Ruxolitinib (P < .001, one-way ANOVA) (Figure S1A), and reduced cell proliferation rates were also apparent during the course of 7 days of Ruxolitinib administration (1 μmol/L) (Figure S1B). Additional cell-cycle analysis experiments further indicated that Ruxolitinib induced a significant decrease of HEL cells in G2 phase (data not shown).

3.2 | Ruxolitinib did not affect healthy donor MSC clonogenicity

Next, we investigated whether the clonogenic capacity of HD-MSC as measured by the CFU-F (colony-forming unit, fibroblast) assay would be affected by JAK2 inhibitor treatment. In a series of 9 independent experiments with MSC from healthy donors, no statistically significant decreases in CFU-F frequency of Ruxolitinib-exposed MSC compared to DMSO-treated controls were observed (Figure 1C). Interestingly, however, increased colony numbers were found at 5 μmol/L Ruxolitinib, that is, the second highest dose tested. In accordance with the proliferation data, colony size measurements showed a trend toward smaller colonies with increasing doses of Ruxolitinib (Figure 1D), but differences were not statistically significant.

**Figure 1** Ruxolitinib effects on in vitro MSC growth and survival. (A, B) Third passage healthy donor (HD) MSC were subjected to short-term (A) and long-term (B) exposure with increasing doses of Ruxolitinib. Complete media changes were performed every 48 h. Data are presented as mean (± SD) relative numbers of viable Ruxolitinib-treated MSC compared to their corresponding DMSO controls (fraction of DMSO controls). Differences were not significant (n = 3 for both short- and long-term exposure, two-way ANOVA) but time was identified as a significant factor for cell survival at 24 h (P = .028, two-way ANOVA). (C) HD-MSC were treated with increasing doses of Ruxolitinib and assayed for clonogenic progenitor cells (CFU-F). Data are shown as mean (± SD) relative number of colonies compared to DMSO-treated controls (n = 9) (fraction of DMSO controls). Colony numbers were significantly higher in the 5 μmol/L Ruxolitinib group compared to the no drug/no DMSO control (*P = .04, unpaired t test). Differences observed at the other dose levels were not significant (ordinary one-way ANOVA, P = .431). (D) Evaluation of colony sizes of Ruxolitinib- and control-treated CFU-F showed a tendency to decreased colony diameters in the Ruxolitinib groups. Bars show the mean (± SD) diameters for the Ruxolitinib- and their respective DMSO-treated cells (n = 5, one-way ANOVA, P = .52). (E, F) Third passage patient (P)-MSC were subjected to short-term (E) and long-term exposure (F) with increasing doses of Ruxolitinib. Complete media changes were performed every 48 h. Data are presented as mean (± SD) relative numbers of viable Ruxolitinib-treated MSC compared to their corresponding DMSO controls (fraction of DMSO controls). Differences were significant for dose in short-term exposure experiments (n = 3, two-way ANOVA, P < .0001), but not in the long-term exposure experiments (n = 2, two-way ANOVA).
3.3 | Ruxolitinib treatment induced a different pattern of proliferation responses in MSC from MPN patients in short-term compared to long-term exposure experiments

Patient MSC were typical spindle-shaped stromal cells, showed a typical CFU-F morphology and were immunophenotypically identical to HD-MSC (Figure S2A-D). Mutational analysis showed that all tested P-MSC and HD-MSC were negative for the JAK2V617F mutation (n = 3 per group). In contrast to HD-MSC, patient MSC (P-MSC) were significantly affected by Ruxolitinib in short-term exposure (48 hours) assays at lower doses (Figure 1E). Here, the most pronounced effect was recorded for the 0.2 μmol/L dose. Interestingly, at the highest Ruxolitinib concentrations (5 μmol/L and 10 μmol/L), P-MSC numbers were not decreased but rather increased, which was in contrast to HD-MSC across all time-points studied (Figure 1A and 1E).

On the other hand, long-term exposure to Ruxolitinib resulted in decreased cell numbers at doses equal to or greater than 0.5 μmol/L after 3 and 7 days of culture (Figure 1F). However, cell numbers recovered to control levels by day 14 despite continuous drug exposure.

3.4 | Ruxolitinib effects on gene expression and JAK-STAT signaling in healthy donor and patient MSC

Next, potential expression differences of genes reported in the context of BM fibrosis and hematopoietic stem cell (HSC) regulation were investigated in P-MSC compared to HD-MSC (Figure 2A-B). Expression levels of fibrosis-related genes lysyl-oxidase 2 (LOXL2), secreted protein acidic and cysteine rich (SPARC), and ADAM metalloepitidase with thrombospondin type 1 motif 4 (ADAMTS4) were higher in the patient samples compared to HD-MSC (Figure 2A). Furthermore, higher expression levels in P-MSC relative to healthy donors were observed for the HSC regulation genes N-cadherin (CDH2) and stromal derived factor-1 (SDF-1/CXCL12) (Figure 2A). Ruxolitinib treatment significantly reduced the expression levels of ADAMTS4 and SDF1, ANGPT1 levels were reduced in 1 of 2 samples and a trend toward reduced gene expression was found for SPARC and CDH2 in P-MSC, whereas expression of LOXL2 was not changed (Figure 2B).

Our MSC proliferation data clearly indicated that Ruxolitinib had considerably less effect on stromal cells compared with JAK2-mutated HEL cells. Therefore, expression levels of JAK2 downstream effectors were investigated in untreated and Ruxolitinib-treated HD-MSC and MSC from patients with JAK2V617F-positive MPN. Phosphorylated STAT3 was considerably reduced in both healthy age-matched MSC and patient MSC exposed to 5 μmol/L of Ruxolitinib for 24 hours (Figure 2C). In addition, phosphorylated AKT (Ser473) levels were lower in the Ruxolitinib-treated samples (Figure 2C).

3.5 | JAK2 inhibition modified the secretory profile of normal and patient MSC

Using the cytokine array as a screening method, the profile of selected cytokines and chemokines was assessed in MSC culture supernatants from healthy donors with and without IL-6 stimulation and subsequent Ruxolitinib treatment (Figure 3A). IL-6 was used in these experiments for JAK-STAT pathway activation. Detectable protein levels were recorded for the following cytokines/chemokines: CD40 ligand, IL-13, IL-16/LCF (Lymphocyte Chemoattractant Factor), MIF (Macrophage migration inhibitory factor), IL-6, IL-23, SerpinE1, MCP-1/CCL2 (Monocyte Chemoattractant Protein-1), IL1Ra/IL-1 F3 (Interleukin 1 Receptor Antagonist), MIP1b/CCL4 (Macrophage Inflammatory Protein 1-Beta), and IFNg (Figure 3A). Among these, cytokines/chemokines with signals above controls (medium control, IL-6 control) and with differences between the IL-6-treated cells and the IL-6 plus Ruxolitinib groups were considered interesting for further evaluation. These included IL-16, IL-1ra, IFNg, MCP-1, IL-23, and IL-6, and as most of them are involved in immunological processes, this qualitative screening assay indicated that the secretome of MSC is altered in response to inflammatory cues, that is, IL-6 stimulation of HD-MSC.

We therefore went on and assessed the levels of selected cytokines and chemokines that were detected in the array and based on their reported association with MPN in the literature. Samples from normal donor and patient-derived MSC were investigated and the data showed that Ruxolitinib induced a significant reduction of MCP-1 and IL-6 levels (Figure 3B,C). Furthermore, we investigated the levels of TGFb1 cytokine and the matrix metalloproteinase MMP2, which are implicated in fibrosis, but their levels remained virtually unchanged (Figure 3D-E).

4 | DISCUSSION

Ruxolitinib is the only JAK inhibitor that is established in routine clinical practice for the treatment of PMF and PV. Ruxolitinib induced remarkable clinical responses on spleen size and MPN-related constitutional symptoms and was demonstrated to prolong overall survival and potentially reverse BM fibrosis.
(A)

Relative fold change

LOXL2  SPARC  ADAMTS4  CDH2  SDF1  ANGPT1

(B)

LOXL2

Relative expression

HD-MSC  P-MSC  P-MSC4  P-MSC5

SPARC

Relative expression

HD-MSC  P-MSC  P-MSC4  P-MSC5

ADAMTS4

Relative expression

HD-MSC  P-MSC  P-MSC4  P-MSC5

CDH2

Relative expression

HD-MSC  P-MSC  P-MSC4  P-MSC5

SDF1

Relative expression

HD-MSC  P-MSC  P-MSC4  P-MSC5

ANGPT1

Relative expression

HD-MSC  P-MSC  P-MSC4  P-MSC5

(C)

kDa

HD-MSC  HD-MSC  P-MSC  P-MSC

+Ruxo  +Ruxo

92

STAT3

86

pSTAT3

60

AKT

60

pAKT (ser473)

42

b-actin
Mesenchymal stromal cells are not only important elements of the normal hematopoietic microenvironment (HME) but also of the malignant stem cell niche. Furthermore, MSC contribute to fibrosis in MF and JAK2, the primary target of Ruxolitinib, is ubiquitously expressed. Thus, the drug is not acting specifically on malignant cells, and it was therefore reasonable to assume that Ruxolitinib would affect also MSC, a question that has not been addressed previously.

The results of the in vitro proliferation experiments showed only moderate direct cytotoxic effects of Ruxolitinib on healthy donor MSC. Interestingly, Ruxolitinib seemed to even promote MSC growth at lower doses (Figure 1A-B) and CFU-F growth was increased at 5 μmol/L, clearly above the clinically observed plasma peak levels. In comparison, JAK2 V617F-positive HEL cell proliferation was clearly inhibited by Ruxo (Figure S1), with an IC50 that was similar to other JAK mutated cell lines. In vitro erythroid colony formation was also reported to be affected by Ruxo (IC50s of 67 nmol/L and 407 nmol/L for patient and healthy donors, respectively). However, was also reported to be affected by Ruxo (IC50s of 67 nmol/L and 407 nmol/L for patient and healthy donors, respectively). However, Ruxo inhibited the growth of CD34 BM cells from JAK2 V617F patients but not normal cord blood controls.

Accordingly, Ruxolitinib showed a trend to affect the proliferation of HD-MSC at higher doses, and CFU-F sizes but not colony numbers were reduced (Figure 1C-D). Patient MSC, on the other hand, showed a somewhat different response to Ruxolitinib (Figure 1E-F). Here, Ruxo seemed to be toxic at lower doses in the short term (Figure 1E), whereas P-MSC growth inhibition was most pronounced at higher doses in long-term exposure experiments (Figure 1F). Interestingly, MSC numbers decreased up to 7 days and recovered to normal values thereafter (Figure 1F). Nevertheless, differences in cell numbers under long-term exposure did not reach statistical significance.

To our knowledge, there are no previous reports on the effects of JAK inhibitors on BM MSC, neither for normal donors nor for patients with JAK2 V617F MPNs. Our data indicate that Ruxolitinib had no or only minor effects on MSC growth at clinically relevant doses, in accordance with the fact that the main MSC proliferation-inducing cytokines only partially signal via JAK-STAT. However, the BM environment in MPNs is characterized by high levels of inflammatory and stromal growth-promoting cytokines that might have primed the patient MSC and thus caused the different response pattern.

We therefore investigated the expression of selected genes, possible JAK-STAT pathway changes and MSC cytokine production profile. P-MSC exhibited higher expression of LOXL2, SPARC, and ADAMTS4 (Figure 2A), which have been reported in the context of BM fibrosis. Furthermore, Ruxolitinib tended to decrease gene expression already after 36 hours of exposure, thus indicating that Ruxolitinib-induced gene expression changes might contribute to the normalization of fibrosis. To our knowledge, fibrosis-related genes have not yet been investigated in Ruxolitinib-treated MPN patients but this would certainly be an important consideration for future studies.

P-MSC also showed a higher expression of hematopoiesis and stem cell-related genes CDH2, SDF-1, and ANGPT1, which decreased when exposed to Ruxolitinib (Figure 2B). Interestingly, CDH2 (N-cadherin) has been reported to be involved in stroma-mediated resistance to tyrosine-kinase inhibition in chronic myeloid leukemia (CML). Increased levels of CDH2 in MPN patients might therefore also be related to therapy resistance in MPNs and thus may represent a possible target for treatment optimization. In accordance with previous reports, SDF1 levels were higher in patient MSC, which is of interest as SDF1 has been implicated in MPN-related stem cell migration and extramedullary hematopoiesis. Angiopoietin-1 (ANGPT-1) is expressed by BM stromal cells and hematopoietic progenitors and was reported to regulate niche regeneration after injury. ANGPT-1 levels were found to be elevated in AML and CML patients. Our data showed that ANGPT-1 displayed higher expression levels also in MPN stroma and might be affected by Ruxolitinib (Figure 2B). However, additional studies are required to more clearly define its role in MPN.

JAK-STAT pathway signaling was clearly inhibited in MSC from JAK2 V617F MPN patients as well as in age-matched HD-MSC (Figure 2C). STAT3 phosphorylation was completely blocked, and downstream AKT phosphorylation (Ser473) was reduced by Ruxolitinib (Figure 2C). Although AKT phosphorylation was reduced, the effect on HD-MSC and P-MSC survival was moderate suggesting that other survival pathways may compensate for this decrease. JAK signaling is constitutively activated in mutated hematopoietic cells in MPN, leading to accumulation of proinflammatory cytokines in the BM and contributing to disease pathology. Ruxolitinib inhibits mutation-induced activated JAK signaling, but BM stromal cells do not belong to the malignant clone (own data and others). Nevertheless, as JAK-STAT signaling is affected by Ruxolitinib even in these cells, we studied the effects of Ruxolitinib on the secretory phenotype of MSC under steady state and under proinflammatory conditions, that is, IL-6 stimulation.

Cytokine array screening identified several cytokines involved in immunological processes that were produced by activated HD-MSC, with some of them responding to Ruxolitinib treatment (Figure 3A). Of these, MCP-1 and IL-6 have been reported to be increased in MPN patients and to correlate with transfusion-dependent anemia and constitutional symptoms, respectively. Both MCP-1 and IL-6 levels in supernatants from P-MSC were reduced by Ruxolitinib (Figure 3B-C). These data therefore indicate that the Ruxolitinib-induced attenuation of the pathologic inflammatory cytokine environment in MPN is not only due to JAK-STAT inhibition of malignant cells but also of non-malignant cells, such as stromal cells.

TGFb signaling pathway is crucial for the development of fibrosis, and we therefore investigated the possible effects of Ruxolitinib on TGFb1 and its activating plasmin protease MMP2 (Figure 3D-E). Neither molecule was affected by Ruxolitinib in our assays suggesting that other cell types than MSC may be more important in this context.

Taken together, we showed that Ruxolitinib affected non-hematopoietic MSC, both from healthy donors and from JAK2 V617F MPN patients. Ruxolitinib did not significantly affect MSC growth.
**Figure 3** JAK2 inhibition modified the secretory profile of MSC. (A) Human cytokine array data from cell culture supernatants of HD-MSC without (HD-MSC control) or with IL-6 stimulation (HD-MSC + IL-6) and subsequent Ruxolitinib treatment (HD-MSC + IL-6 + RUXO). Data are presented as mean ±SD (qualitative data from n = 3, biological replicates from 3 independent experiments, a.u.: arbitrary units, b.d.: below detection). Medium control: medium only; IL-6 control: medium supplemented with 100 ng/mL IL-6. Chemokine/cytokine concentrations of MCP-1 (P = .0012) (B) and IL-6 levels (P < .0001) (C), TGFb1 (D) and matrix metalloproteinase MMP-2 (E) in cell culture supernatants of HD-MSC and patient MSC (patients P-MSC4-6) after Ruxolitinib (1 μmol/L) exposure for 24 h. Data are presented as mean ± SD in B-C (n = 3 P-MSC, n = 1 HD-MSC, data from 2 independent experiments) and mean ± CV from technical duplicates in D-E (n = 2 P-MSC, n = 3 HD-MSC). Significant differences are indicated as ****P < .0001, **P = .005 (one-way ANOVA with Tukey’s multiple comparisons test). MCP-1 and IL-6 levels were measured by ELISA and single-plex assays were used for TGFb1 and MMP-2 quantification.
in a direct cytotoxic manner, but did cause gene expression changes and altered the paracrine signaling of MSC in vitro. These data thus suggest that the therapeutic benefit of Ruxolitinib may be partly attributed to MSC signaling alterations in the inflammatory milieu of MPNs. Therefore, future studies on a larger number of patients investigating the in vivo role of stromal contributions in MPN pathogenesis are certainly highly motivated and have the potential to identify novel targets for the development of more effective treatments in myeloproliferative disorders.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

D.Z. designed and performed experiments, and wrote the manuscript. R.G., H.L., and H.C.L. performed experiments, S.S. designed D.Z. designed and performed experiments, and wrote the manuscript. All authors contributed to data analysis and interpretation, and critically reviewed the manuscript.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.