A high-content cytokine screen identifies myostatin propeptide as a positive regulator of primitive chronic myeloid leukemia cells

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ABSTRACT

Aberrantly expressed cytokines in the bone marrow (BM) niche are increasingly recognized as critical mediators of survival and expansion of leukemic stem cells. To identify regulators of primitive chronic myeloid leukemia (CML) cells, we performed a high-content cytokine screen using primary CD34+CD38low chronic phase CML cells. Out of the 313 unique human cytokines evaluated, 11 were found to expand cell numbers ≥2-fold in a 7-day culture. Focusing on novel positive regulators of primitive CML cells, the myostatin antagonist myostatin propeptide gave the largest increase in cell expansion and was chosen for further studies. Herein, we demonstrate that myostatin propeptide expands primitive CML and normal BM cells, as shown by increased colony-forming capacity. For primary CML samples, retention of CD34-expression was also seen after culture. Furthermore, we show expression of MSTN by CML mesenchymal stromal cells, and that myostatin propeptide has a direct and instant effect on CML cells, independent of myostatin, by demonstrating binding of myostatin propeptide to the cell surface and increased phosphorylation of STAT5 and SMAD2/3. In summary, we identify myostatin propeptide as a novel positive regulator of primitive CML cells and corresponding normal hematopoietic cells.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm caused by an acquired 9;22-chromosomal translocation in a hematopoietic stem cell (HSC) resulting in the expression of the BCR-ABL1 fusion protein.1 The BCR-ABL1 fusion protein is a constitutively active tyrosine kinase and triggers a cascade of aberrant downstream signaling pathways leading to clonal outgrowth of CML cells and subsequent disease manifestation.1,2 There is growing evidence to suggest that primitive CML cells affect the bone marrow (BM) niche, contributing to deregulated cytokine levels.3 In CML, several pro-inflammatory cytokines, such as IL-6,4 IL-18,5 and TNF-α,6 have been shown to be up-regulated in patient serum. Cytokines are essential for the function and maintenance of cells, and altered cytokine levels influence not only leukemic cells, but also the normal HSC within the BM. A pro-inflammatory environment is thought to provide a selective advantage for the leukemic stem cells (LSC).7 In CML and acute myeloid leukemia (AML), we and others have shown that IL-1 is a positive regulator of LSC, and blocking IL-1 signaling inhibits the LSC.8,9 By contrast, chronic exposure to IL-1...
leads to exhaustion of normal HSC.11 Therefore, inhibition of the pro-inflammatory environment in the disease might have therapeutic potential.7 Hence, a better understanding of the autocrine and paracrine signaling important for LSC survival and maintenance will not only be of great importance for characterizing disease biology and progression, but might also translate into the development of novel therapies targeting the LSC.

To identify key positive regulators of CML stem cells, we conducted a high-content cytokine screen on stem cell enriched primary chronic phase CML cells using an arrayed library of 313 unique human cytokines. This screen confirmed the positive regulatory effect of IL-3,12,13 IL-1α/β,16–18 GM-CSF,19 IL-6,19,20 and IFN-γ,21 cytokines previously reported to expand primitive CML cells, and also identified several novel positive regulators. Among the novel positive regulators, we identified myostatin propeptide (MSTNpp), a muscle secreted protein not previously implicated in the regulation of normal or malignant hematopoiesis, and demonstrate that MSTNpp promotes the growth and survival of both primitive CML and normal hematopoietic cells.

Methods

Patient samples and CD34 enrichment

Bone marrow and peripheral blood (PB) from untreated chronic phase CML patients, AML patients or blast crisis CML patients were obtained after written informed consent and in accordance with the Declaration of Helsinki. The Regional Ethics Committee (Dnr 2017/391) approved the study. All cellular chronic phase CML samples included in the study are summarized in the Online Supplementary Table S1. For information on cell preparation, see the Online Supplementary Methods.

Cytokine screening

Chronic myeloid leukemia PB samples with high IL1RAP expression within the CD34+CD38low compartment, correlating with a high fraction of BCR-ABL1 positive cells,15,16 were selected for the screen (Online Supplementary Figure S1). IL1RAP expression in the CD34+CD38low cells was assessed by flow cytometry as previously described.19 In brief, 500 CD34+CD38low cells were sorted into pre-prepared cytokine plates containing 313 unique cytokines, and cell numbers were evaluated after seven days. For detailed information on cytokine library preparation, antibody staining, cell sorting, experimental read out and screen validation, see the Online Supplementary Methods.

Cell cultures

In all cell culture experiments, cells were cultured in serum free StemSpan™ SFEM media (Stem Cell Technologies, Canada). Cell numbers were evaluated using CountBright Absolute Counting Beads (Thermo Fisher Scientific, USA) and Draq7 (BioStatus, UK) on a LSR Fortessa (BD Biosciences, USA).

Cell cultures of transgenic BCR-ABL mouse cells

Transgenic ScltTA/BCR-ABL mice22 were taken off tetracycline pellets to induce CML-like disease. Leukemic mice and age-matched wild-type B6.SJ mice were sacrificed 8-10 weeks post induction. Five thousand Lin−Sca-1−c−Kit− (LSK) BM cells were sorted into individual wells of a 96-well plate containing 500 ng/mL of MSTNpp (catalog# 12012, lot# 0605297, Peprotech) or no cytokine control, and cell numbers were analyzed after seven days. For detailed information on cell isolation, antibody staining, sorting, and cell culture see the Online Supplementary Methods.

Colony-forming assays

CD34+ chronic phase CML cells, CD34+CD38low chronic phase CML cells, or equivalent normal BM cells, were cultured for a week with or without cytokine, prior to transfer to H4435 MethoCult (Stem Cell Technologies) according to the manufacturer’s instructions. See the Online Supplementary Methods for details on readout and colony replating.

Co-culture experiments with primitive chronic myeloid leukemia cells and mesenchymal stromal cells

CD34+CD38low CML cells were sorted as described above, and plated onto mesenchymal stromal cells (MSC) established from primary CML BM cells in a 1:5 ratio. Details on MSC cultures and culture conditions can be found in the Online Supplementary Methods.

MSTNpp binding experiment

KU81221 cells were resuspended in PBS with 2% FBS. Two ug/mL of MSTNpp (catalog# 12012, lot# 0605297, Peprotech) or no cytokine was added together with a polyclonal rabbit IgG anti-MSTNpp antibody (Antibodies-online, USA), and stained on ice in the dark for 30 minutes (min). Cells were washed twice in PBS with 2% FBS, resuspended in goat anti-rabbit BV421 (BD Biosciences) and stained on ice in the dark for another 30 min. After a final wash, cells were analyzed on a LSRFortessa (BD Biosciences). Draq7 (BioStatus) was used as a viability marker.

MSTNpp ELISA

MSTNpp concentrations in PB plasma samples from 12 CML patients and four healthy donors, and BM plasma from five healthy donors, were diluted 1:20 and analyzed using Human Myostatin ELISA (prodomain specific) (BioVendor, Czech Republic) according to the manufacturer’s instructions.

MSTN reverse transcriptase-quantitative polymerase chain reaction

Relative MSTN expression in CD34+ cells, MNC and MSC from CML BM was assessed using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). For detailed methodology and assays used, see the Online Supplementary Methods.

RNA sequencing and gene set enrichment analysis

Regarding the gene set enrichment analysis (GSEA), CD34+CD38low (lowest 5% of CD38-expressing cells) cells from PB of five chronic phase CML patients were sorted into flat bottom 96-well plates with or without 500 ng/mL of MSTNpp (Peprotech), with cell numbers ranging from 2,000-11,000 cells, and incubated for 3 h or 24 h. For detailed information on RNA-extraction, cDNA synthesis, library preparation, sequencing and analysis pipeline, see the Online Supplementary Methods.

Phospho-flow cytometry

Cells were resuspended in serum free StemSpan™ SFEM medium (Stem Cell Technologies) and incubated for 3 h at 37°C 5% CO2, prior to 15 min stimulation with 500 ng/mL of MSTNpp (Peprotech) or 10 ng/mL of TGF-β1 (Peprotech). Following fixation with 1.6% paraformaldehyde for 10 min at room temperature and permeabilization with 90% ethanol at -80°C, cells were stained with phospho-specific antibodies. Details on staining and the antibodies used are described in the Online Supplementary Methods.
Statistical analysis
All bar graphs show mean values and standard deviation. Differences between groups were assessed by unpaired Student’s t-test using Prism 6 software (GraphPad Software, USA).

Results
Cytokine screening identifies MSTNpp as a positive regulator of CD34^+CD38low chronic myeloid leukemia cells
To identify positive regulators of primitive CML cells, we established a high-content cytokine screen using 313 unique human cytokines (Online Supplementary Table S2). CD34^+CD38low chronic phase CML PB samples with high leukemic burden (Online Supplementary Figure S1) were sorted into 384-well plates with one cytokine condition per well. After a 7-day culture, the number of live cells was enumerated with automated fluorescence microscopy (Figure 1A).

In total, we identified 11 cytokines that increased cell numbers at least 2-fold compared to a no cytokine control (Figure 1B). Of these, the cytokines IL-3, IL-1α/β, GM-CSF, IL-6, and IFN-γ have previously been described as positive regulators of CML stem and progenitor cells, thus validating the robustness of the screen. Notably, five cytokines not previously described as positive regulators of CML stem and progenitor cells were identified: MSTNpp, soluble CD14 (sCD14), Interleukin 21 (IL-21), Interleukin 13 variant (IL-13v), and chemokine...
(C-C motif) ligand 28 (CCL28). To investigate whether the positive regulators identified also affected CD34+CD38low CML cells from BM, we performed a more focused screen using only the top regulating cytokines in a 96-well format. All cytokines identified as positive regulators of primitive CML PB cells also increased the number of primitive CML BM cells (Figure 1C). However, only IL-3, IL-1α/β, GM-CSF, IL-6, MSTNpp, and IFN-γ were able to expand cell numbers at least 2-fold, which was used as the cut-off in the original screen.

We next investigated whether the positive regulators identified also expanded normal CD34+CD38low BM cells, enriched for HSC (Figure 1D). Whereas IL-3 expanded the normal BM cells during a 7-day culture, none of the other cytokines increased the total cell number. However, MSTNpp promoted their survival, as indicated by a higher cell number compared to the no cytokine control.

**MSTNpp increases the progenitor potential of chronic myeloid leukemia and normal bone marrow cells**

Of the top regulators identified, we selected MSTNpp for further studies given its strong effect on primary CML cells and its previously unexplored role in normal and malignant hematopoiesis. MSTNpp is produced by muscle cells and secreted into the bloodstream where its recognized function is to bind and regulate the muscle inhibiting myokine myostatin (also known as GDF-8). To study the growth dynamics of CML cells in response to different concentrations of MSTNpp, a dose titration experiment was performed on CD34+ CML cells. In a 7-day culture, MSTNpp increased the cell number in all treated wells in a dose-dependent manner (Figure 2A), with similar results observed during three days of culture (Online Supplementary Figure S2A). Adding a polyclonal anti-MSTNpp antibody to the culture reduced the growth promoting effect of MSTNpp on CML cells, confirming the specificity of the observed response (Figure 2B).

To investigate whether MSTNpp had a positive effect also on primitive murine CML cells, Lin’ Sca-1+ c-Kit+ (LSK) BM cells from transgenic Scl-tTA/BCR-ABL mice were cultured in vitro with or without MSTNpp. In a 7-day culture, MSTNpp greatly increased the cell number compared to untreated cells (Figure 2C). For wild-type murine LSK BM cells from B6.SJL mice, MSTNpp nearly maintained the cell number, hence promoting cell survival compared to no cytokine control (Figure 2D). These results are in agreement with our findings using human CD34+CD38low chronic phase CML PB and BM cells (Figure 1B and C), as well as for normal CD34+CD38low BM cells (Figure 1D).

Given the robust response of MSTNpp in chronic phase CML, we hypothesized that MSTNpp could have a similar growth promoting effect on other myeloid malignancies. Therefore, we cultured primary blasts from five AML patients and three CD34+ blast crisis CML patients with and without MSTNpp, and evaluated the cell numbers at day 3 or 4 and day 7 (Online Supplementary Figure S2B and C). Out of the five AML patients tested, two responded to MSTNpp with a slight increase in survival at day 3. At day 7, however, this effect was no longer seen. Another AML patient showed a response to MSTNpp only at day 7, but not at day 3. For the CD34+ blast crisis CML cells, one out of the three samples responded to MSTNpp stimulation with increased survival; the other two, including a sample with a T315I-mutation, did not respond. These results suggest that, unlike chronic phase CML cells, blast crisis CML cells and AML blasts are not consistent in their response to MSTNpp.

Next, we investigated the possible effects of MSTNpp on cellular differentiation of primary human CD34+CD38low CML cells. Since CD34 expression, in contrast to CD38 expression, correlates with stem cell activity of in vitro cultured cells, IL-3 and CD34+CD38low cells were cultured with or without MSTNpp for seven days in serum free media, prior to plating in methylcellulose media. MSTNpp significantly increased the number of CML colonies compared to control cells without cytokine (Figure 2F). Moreover, pre-stimulation with MSTNpp increased the colony output upon replating (Figure 2F), suggesting that MSTNpp expands CML cells with self-renewal capacity.

We also investigated the effects of MSTNpp on the colony forming ability of normal CD34+CD38low BM cells after one week in culture. Although MSTNpp did not expand normal CD34+CD38low BM cells during a 7-day culture (Figure 1D), MSTNpp had a marked effect on the subsequent colony forming ability of the stimulated cells as compared to no cytokine control (Figure 2G and Online Supplementary Figure S2D). These findings show that MSTNpp promotes the colony forming capacity of both CML cells and normal hematopoietic stem and progenitor cells (HSC).

Finally, to study the effect of MSTNpp on primitive CML cells in a setting that more closely resembles the BM microenvironment, we performed co-culture experiments where CD34+CD38low CML cells were cultured on a monolayer of MSC established from primary CML BM (Figure 2H). When cultured on stroma, there were twice as many CML cells in the culture after three days, compared to the no stroma control. This suggests that the CML MSC produce growth factors important for the growth and survival of primitive CML cells. When adding MSTNpp to the co-culture, the cell numbers increased even further (approx. 2-fold), showing that primitive CML cells respond to MSTNpp also in a setting where other stimuli are present. These findings suggest that the effect of MSTNpp on CML cells is physiologically relevant.

**MSTNpp affects chronic myeloid leukemia cells through mechanisms independent of myostatin**

Myostatin is a transforming growth factor beta (TGF-β) superfamily member, synthesized as part of the myostatin gene (MSTN) and produced primarily by muscle cells. The MSTN gene encodes a biologically-inactive precursor protein consisting of a signaling peptide, the MSTNpp-domain and the myostatin-domain. Proteolytic
Myostatin propeptide expands primitive CML cells

Figure 2. MSTNpp increases progenitor potential of chronic myeloid leukemia (CML) and normal bone marrow (BM) cells. (A) Bar graph showing total cell numbers of CD34+ chronic phase CML cells from a single patient after 7-day culture with increasing concentrations of MSTNpp. The dotted line indicates the number of seeded cells at day 0 (n=3). (B) Bar graph showing total cell numbers after culture of 100 ng/mL of MSTNpp with a polyclonal anti-MSTNpp antibody. The same cells and culturing conditions as in (A) were used (n=3). (C) Bar graph showing absolute cell numbers of Lin-Sca-1-c-Kit+ (LSK) BM cells from ScltTA/BCR-ABL mice after 7-day culture with or without 500 ng/mL of MSTNpp (n=3). (D) Bar graph showing total cell numbers of LSK BM cells from WT B6.SJL mice after 7-day culture with or without 500 ng/mL of MSTNpp (n=3). (E) Representative histograms showing CD34 expression of CD34+CD38 low chronic phase CML BM cells after a 7-day culture with indicated cytokines. (F) Colony type and number of CD34+ and CD34+CD38 low CML cells, pre-cultured for seven days with or without 500 ng/mL of MSTNpp prior to plating in methylcellulose. A cell equivalent of 500 starting cells was plated. Three individual patient samples were used in triplicates. Replating was performed after two weeks in colony culture for one of the samples. No colonies were seen after the 3rd replating. (G) Colony type and number of CD34+CD38 low normal BM cells using the same experimental setup as in (F). Cells from two normal donors were used in triplicates. (H) Bar graph showing total cell numbers of CD34+CD38 low CML cells after 3-day culture with and without 100 ng/mL of MSTNpp. Gray bars: cells cultured in suspension; black bars: cells co-cultured with CML mesenchymal stromal cells (MSC). Two individual patient samples were used in triplicates. The dotted line indicates the number of seeded cells. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. WT: wild type.
The cleavage of the precursor protein generates active and circulating MSTNpp and myostatin. Active myostatin is antagonized by MSTNpp by forming a latent complex which prevents binding of myostatin to its receptor (Figure 3A). To explore the mechanism by which MSTNpp exerts its growth promoting effects on CML cells, we first investigated if MSTNpp acts by blocking the activity of myostatin. CML CD34+ cells were cultured for seven days with or without myostatin and MSTNpp (Figure 3B). No difference in cell number was seen between myostatin cultured cells as compared to a no cytokine control, whereas MSTNpp, serving as a positive control, increased the total cell number. This finding indicates that myostatin does not exert a negative effect on CML-progenitor cell growth, suggesting that MSTNpp does not promote CML cells by inhibiting myostatin, but through another mechanism.

To investigate the possibility that MSTNpp acts directly on CML cells by binding to a receptor on the cell surface, KU812 cells were incubated with an anti-MSTNpp antibody together with MSTNpp, or with anti-MSTNpp antibody only, and analyzed by flow cytometry. MSTNpp was found to bind to the cell surface, as a shift in fluorescence intensity was observed when both MSTNpp and the anti-MSTNpp antibody were added to the cells (Figure 3C).

**MSTNpp is produced by chronic myeloid leukemia mesenchymal stromal cells and is present in the plasma of chronic myeloid leukemia patients**

It is known that MSTNpp is produced by muscle cells and is present in the blood of healthy individuals. To investigate whether the leukemic cells or the MSC within the marrow are another source of MSTNpp, we performed a *MSTN* RT-qPCR on CD34+ cells and MNC from primary CML patients, as well as on cultured MSC from

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**Figure 3. MSTNpp acts through mechanisms independent of myostatin in chronic myeloid leukemia (CML) and is produced by CML mesenchymal stromal cells (MSC).** (A) Schematic illustration showing the MSTN precursor protein before and after enzymatic cleavage, generating MSTNpp and myostatin. (B) Bar graphs showing total cell numbers of CD34+ chronic phase CML cells after 7-day culture with 100 ng/mL of myostatin, 100 ng/mL of MSTNpp or no cytokine control. Two individual patient samples were used in triplicates. (C) Histogram showing binding of MSTNpp to the surface of KU812 cells, as compared to no cytokine control. (D) Bar graph showing relative *MSTN* gene expression of CD34+ cells, mononuclear cells (MNC) and cultured MSC from three chronic phase CML patients, in relation to expression in human skeletal muscle. (E) MSTNpp concentration in plasma of peripheral blood (PB) from 12 chronic phase CML patients, PB from four healthy donors and bone marrow (BM) from five healthy donors. The middle line in each bar indicates the mean plasma concentration. *P<0.05; **P<0.01.
the same patients (Figure 3D). All cell fractions tested expressed the MSTN gene, with the highest expression in MSC. Although the expression levels were approximately 20x lower in MSC than in skeletal muscle, these data suggest that the MSC in the BM are another source of MSTNpp, possibly contributing to the growth and survival of CML cells.

To evaluate whether the MSTNpp levels are different in leukemic and healthy individuals, we performed a MSTNpp specific ELISA on plasma from CML patients and healthy individuals (Figure 3E), as well as on plasma from healthy BM aspirates. All samples showed detectable levels of MSTNpp, but there was no significant difference between MSTNpp levels in CML patients versus normal individuals. Moreover, the concentrations of MSTNpp varied greatly depending on the patient/donor, with levels ranging from ~0.1-400 ng/mL. As CML cells respond to MSTNpp even at 1 ng/mL (Figure 2A), the MSTNpp concentrations in the plasma from CML patients are within physiologically relevant levels for the leukemic cells.

**MSTNpp stimulation activates STAT5 and SMAD2/3 in chronic myeloid leukemia cells**

To search for cellular programs and signaling pathways transcriptionally regulated by MSTNpp in primitive CML...
cells, we performed RNA-sequencing following 3-h and 24-h culture of CD34+CD38\textsuperscript{low} cells with or without MSTNpp (Figure 4A). After 3 h, 147 genes were significantly up-regulated and 115 genes were significantly down-regulated in the MSTNpp stimulated cells compared to the non-stimulated control cells (FDR<0.01) (Online Supplementary Table S3). After 24 h, there were fewer differentially expressed genes, with 56 up-regulated genes and only 14 genes down-regulated (FDR<0.01) (Online Supplementary Table S3). To explore whether MSTNpp stimulation activates specific downstream pathways in CML cells, we performed GSEA. Following 3 h stimulation with MSTNpp, an enrichment of STAT5 target genes was observed (Figure 4B). In addition, MSTNpp, which is a TGF-β superfamily member, induced a significant enrichment of TGF-β1 target genes both after 3 h and after 24 h (Figure 4B and Online Supplementary Figure S3A). After 24 h, genes associated with cell cycle and DNA-replication were also enriched (Online Supplementary Figure S3A), consistent with the increased proliferative response seen after stimulation by MSTNpp (Figures 1B and C and 2A and Online Supplementary Figure S2A).

Given that MSTNpp stimulation resulted in enrichment of STAT5 and the TGF-β1 target genes, we performed phospho-flow cytometry of STAT5 and SMAD2/3 in CD34\textsuperscript{+} chronic phase CML cells. Already 15 min following MSTNpp stimulation, we observed an increase in the phosphorylation levels of STAT5 and SMAD2/3 (Figure 4C and D and Online Supplementary Figures S3B and S4). Interestingly, the MSTNpp-induced activation of SMAD2/3 was similar to that of TGF-β1 (Online Supplementary Figure S3C), one of the well-known activators of SMAD2 and SMAD3.\textsuperscript{24} However, culturing CD34\textsuperscript{+} CML cells with TGF-β1 reduced cell numbers (Online Supplementary Figure S3D), whereas MSTNpp stimulation resulted in growth-promoting effects (Figure 2A and Online Supplementary Figure S2A). When an additional panel of phospho-antibodies was tested to evaluate changes in MAPK-pathways (pERK, pJNK and pERK1/2),\textsuperscript{25} IL-1 signaling (NF-κB and pAkt),\textsuperscript{26,27} and general phosphotyrosine activation (pTyre)\textsuperscript{28} (Online Supplementary Figure S4), signaling pathways associated with cell proliferation and cancer,\textsuperscript{28,29} no changes were detected for any of these markers.

Discussion

Deregulation of cytokines in the BM niche is thought to play a major role in leukemic disease progression, with altered cytokine levels promoting the growth of LSC while suppressing normal HSC.\textsuperscript{7,35,36} In CML, a transgenic BCR-ABL\textsubscript{1} mouse model revealed extensive remodeling of the BM along with altered cytokine and chemokine levels, secreted both by proximal stroma cells and leukemic cells.\textsuperscript{37} CML cells also produce and respond to autocrine stimulation by IL-3 and granulocyte-colony stimulating factor (G-CSF),\textsuperscript{37} and recent work by our group and others has highlighted IL-1 as a positive regulator of CML LSC.\textsuperscript{48} Hence, increased understanding of the cytokine-receptor interactions and signaling pathways activated in the immature cell population of both CML and normal BM cells might reveal disease dependencies that could translate into new treatment opportunities in CML and other malignancies.

We here conducted a high-content cytokine screen with the purpose of finding novel positive regulators of primitive CML cells. Previous studies have mainly investigated the effects on CML cells of single or a restricted set of cytokines.\textsuperscript{5,9,15,38,39} With a library of 313 cytokines screened, we identified 11 cytokines that at least doubled the cell number over seven days compared to no cytokine control. The screen confirmed the growth-promoting effect of IL-3,\textsuperscript{12,13} IL-1α/β,\textsuperscript{3} GM-CSF,\textsuperscript{3} IL-6,\textsuperscript{15,16} and IFN-γ\textsuperscript{40} for CD34\textsuperscript{+}CD38\textsuperscript{low} PB and BM chronic phase CML cells. The screen also identified five cytokines not previously reported to be important for CML; MSTNpp, sCD14, IL-21 and IL-15v, and CCL-28. Out of these novel cytokines, the TGF-β superfamily member MSTNpp was the most potent in promoting the growth of primitive CML cells. Previously, most studies of MSTNpp had been carried out in the context of muscle physiology where it regulates the muscle inhibiting myokine myostatin.\textsuperscript{24,40} By binding free myostatin in the blood, MSTNpp hinders myostatin from binding to activin receptors and activating muscle wasting programs in the cell.\textsuperscript{24,40} Recently, it was also shown that MSTNpp can bind directly to activin receptors on muscle cells, blocking access by myostatin, and thus providing another mechanism of myostatin inhibition.\textsuperscript{40} No studies describing MSTNpp effects independent of myostatin have been previously reported. However, our data strongly suggest that MSTNpp has a direct effect on hematopoietic cells, by binding to a receptor on the cell surface independently of myostatin. This conclusion is based on the following observations; the in vitro screen was performed in serum-free media with no myostatin present, and addition of myostatin had no adverse effects on the growth of CML cells. Therefore, the mechanism by which MSTNpp acts in normal and malignant hematopoiesis seems to differ from what has previously been described in muscle cells. Further, we confirmed that MSTNpp is present in the plasma of CML patients and normal individuals, and demonstrate for the first time that MSC, MNC and CD34\textsuperscript{+} cells from CML patients express MSTN. Out of these cell types, MSC expressed the highest levels of MSTN. Importantly, even though there was no difference in MSTNpp plasma concentration between the two groups, primitive CML cells are more responsive to MSTNpp stimulation than corresponding normal cells.

MSTNpp stimulation of chronic phase CML CD34\textsuperscript{+}CD38\textsuperscript{low} cells greatly increased the number of cells in culture without loss of CD34-expression, suggesting that MSTNpp expands the primitive cells while keeping them in an immature state. This finding was further strengthened by colony-forming assays, where MSTNpp pre-stimulation increased the number of colonies of both CML and normal BM cells. This indicates that the growth promoting effect of MSTNpp is not restricted to CML cells, but also applies to normal hematopoietic stem and progenitor cells (HSPC). Whether MSTNpp would elicit a differential regulatory effect on primary CML LSC compared to normal HSC in vivo is difficult to assess, given that primary CML cells engraft poorly in immunodeficient mice.

The observation that MSTNpp stimulation expanded primitive CML cells and resulted in the activation of both STAT5 and SMAD2/3 is intriguing, as these signaling pathways are associated with different cellular effects. By
stimulation with TGF-β1, we found SMAD2/3 phosphorylation as well as reduced growth and survival of primary CD34+ CML cells. This suggests that MSTNpp-induced SMAD2/3 activation is an unlikely cause of the growth-promoting effects. Instead, the increased STAT5 phosphorylation is a probable mechanism by which MSTNpp expands primitive CML cells. Although STAT5 phosphorylation was high in the unstimulated control cells (consistent with STAT5 being a well-known mediator and downstream target of the BCR-ABL1 fusion in CML), MSTNpp stimulation further activated STAT5. However, cross-talk between STAT5, TGF-β family signaling members and other signaling pathways might also contribute to the growth-promoting effects of MSTNpp in primitive CML cells.

In conclusion, we hereby identify several novel positive regulators of primitive CML cells using a high-content cytokine screen. We show that the myostatin antagonist MSTNpp binds to the surface of CML cells, induces activation of STAT5 and SMAD2/3, and has a previously unrecognized recognition of growth-promoting effects on primitive CML cells and corresponding normal cells. Further studies are needed to investigate whether interfering with MSTNpp would translate into new therapeutic opportunities in CML.

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