Lipocalin Produced by Myelofibrosis Cells Affects the Fate of Both Hematopoietic and Marrow Microenvironmental Cells

Running title: Lipocalin Affects Both HPC and Stromal Cells

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**Key point 1:** LCN2 levels are elevated in MF plasma and acts to generate ROS leading to increased DNA strand breaks and apoptosis in normal CD34\(^+\) cells

**Key point 2:** LCN2 promotes the generation of osteoblasts but diminishes adipogenesis resembling the composition of the MF marrow microenvironment
Abstract

Myelofibrosis (MF) is characterized by cytopenias constitutional symptoms, splenomegaly and marrow histopathological abnormalities (fibrosis, increased microvessel density and osteosclerosis). The microenvironmental abnormalities are likely a consequence of the elaboration of a variety of inflammatory cytokines generated by malignant megakaryocytes and monocytes. We observed that levels of a specific inflammatory cytokine, lipocalin-2 (LCN2) were elevated in the plasmas of patients with myeloproliferative neoplasms (MF > PV or ET) and that LCN2 was elaborated by MF myeloid cells. LCN2 generates increased reactive oxygen species leading to increased DNA strand breaks and apoptosis of normal but not MF CD34+ cells. Furthermore, incubation of marrow adherent cells or mesenchymal stem cells with LCN2 increased the generation of osteoblasts and fibroblasts but not adipocytes. LCN2 priming of MSCs resulted in the upregulation of RUNX2 gene as well as other genes which are capable of further affecting osteoblastogenesis, angiogenesis and the deposition of matrix proteins. These data indicate that LCN2 is an additional MF inflammatory cytokine which likely contributes to the creation of a cascade of events that result not only in predominance of the MF clone but also a dysfunctional microenvironment.
Introduction

Cross-talk between hematopoietic cells with non-hematopoietic marrow cells in myelofibrosis (MF) contributes to distinctive marrow micro-environmental changes which likely comprise the function of specific marrow niches which support normal hematopoiesis (1-3). MF cells elaborate cytokines which contribute to the development of marrow fibrosis, increased microvessel density and osteosclerosis (3). These cytokines affect marrow mesenchymal cells that are not involved by the malignant process (3-9).

Recently, neutrophil gelatinase-associated lipocalin (LCN2) has been implicated in the pathobiology of myeloproliferative neoplasms (MPNs)(10-13). LCN2 promotes the proliferation of the malignant clone in chronic myeloid leukemia (14). In addition, LCN2 gene expression has been reported to be increased in CD34^+ cells isolated from primary MF (PMF) as well as PV patients while LCN2 levels were elevated in the plasma of MPN patients (10-13). Furthermore, Kagoya and coworkers in a mouse model demonstrated that $JAK2V617F$ positive cells elaborate LCN2 resulting in DNA damage in neighboring normal cells as well as cells belonging to the malignant clone by generating reactive oxygen species (11). We, therefore, further examined the role that LCN2 might play in MPNs.
Material and Method

Specimen collection

Patient samples were collected at the Icahn School of Medicine at Mount Sinai (ISMMS). Written informed consent was obtained from patients according to guidelines established by the Institutional Review Board of the ISMMS, New York, NY. All patients met the World Health Organization diagnostic criteria for polycythemia vera (PV), essential thrombocytemia (ET) and PMF (1). CD34+ cells were isolated using a human CD34+ cell selection kit (StemCell Technologies, Vancouver, BC, Canada), T and B cell depleted MNC were isolated using a human lymphocyte selection kit (StemCell Technologies). The severity of MF in 70 patients was assessed using prognostic parameters utilized for the development of the Dynamic International Prognostic Scoring System (DIPPS)(15-16).

Preparation of BM adherent cells

Human BM mononuclear cells (MNC) were cultured in mesenchymal stem cell growth medium (MSCGM) (Lonza) at a density of 2-5X10⁴/ml in culture flasks or dishes. The cells that remained in suspension were removed by completely changing the medium at the time adherent cell (AC) layers were formed. The medium was changed twice weekly by replacing half of the medium with fresh MSCGM. Studies were performed with ACs that had not been passaged more than 4 times.

Cell proliferation assays
CD34+ cells were cultured with Serum-Free Medium (SFEM) (StemCell Technologies) containing stem cell factor (SCF), thrombopoietin (TPO), fms-like tyrosine kinase 3 ligand (Flt-3L) and interleukin-3 (IL-3) (R&D Systems Minneapolis, MN) with and without LCN2 (Sigma, St. Louis, MO). Cell viability was determined using trypan-blue (Sigma). The proportion of CD34+ cells was measured flow cytometrically after staining with an anti-CD34 antibody (BD Biosciences, San Jose, CA). AC proliferation was determined using the PrestoBlue cell viability reagent (Life Technologies, CA).

**JAK2V617F genotyping of hematopoietic colonies**

CD34+ cells were plated in 30mm dishes containing 1 mL SFEM with 1.1% methylcellulose, to which SCF, TPO, Flt-3L, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-3 and EPO were added with or without LCN2 (17). Individual colonies were randomly plucked and JAK2V617F was detected using nested allele-specific PCR (17).

**Flow cytometric analyses**

Cells were collected and washed with MACS buffer, and were stained with anti-CD34 antibody, annexin-V (BD Biosciences) or LCN2 receptor antibody (Abcam, Cambridge, MA) directly. For intracellular staining, cells were fixed with 4% formaldehyde and permeabilized, and then stained with antibody to γH2AX, or adichlorofluoresendiacetate (DCFDA) (Abcam) to evaluate the ROS activity. Data were acquired using a FACS Caliber analyzer (BD Biosciences).

**Immunofluorescent and immunohistochemical staining**

Cells were fixed with 4% formaldehyde, permeabilized, and then stained with primary antibodies. The primary antibodies were visualized with AlexaFluor 546- or AlexaFluor 488-
conjugated IgG (Life technologies). Stained slides were mounted using ProLong® Gold Anti-fade Reagent (Life Technologies, Norwalk, CT). Fluorescent images were acquired using a 1X71 fluorescence microscope (Olympus Corporation, Tokyo, Japan) and MicroSuite software (Olympus). Sections of formalin fixed and paraffin-embedded MF marrow biopsies were baked and deparafinized. Immuno-histochemical staining of LCN2 (Abcam) was performed using the Bond III Autostainer (Leica Microsystems, Illinois). The degree of fluorescence intensity was assessed using MetaMorph® Microscopy Automation & Image Analysis Software (Molecular Devices, Sunnyvale, CA).

BM mesenchymal stem cell differentiation assays

BM AC cells were cultured in MSCGM with or without LCN2 for at least ten days, and then with media designed to favor either adipogenic or osteogenic differentiation (R & D Systems). The cells then were fixed and underwent immunostaining.

Isolation of RNA and qRT-PCR

BM ACs were cultured with MSCGM alone or medium containing LCN2 for 1 to 10 days. Total RNA was extracted from the ACs using RNeasy kit (Qiagen, Valencia, CA). cDNA was reverse transcribed using EcoDry Premix kit (Clontech). The BMP2, COL1A1, OPG, PPARγ, RUNX2, TGF-β, VEGF and GAPDH genes were evaluated by qPCR which was performed using SYBR® Green-based RT² qPCR Master Mixes from Qiagen and the Realplex thermocycler (Eppendorf).

Statistical methods

Non-parametric Wilcoxon Rank Sum Tests were used to assess two group differences; non-parametric Kruskal Wallis analysis of variance F tests were used to assess multi-group
differences. Correlations between LCN2 and hematologic parameters were determined using non-parametric Spearman correlation coefficients. ANOVA F-tests were used to evaluate differences between cell samples. All pairwise comparisons were performed with Sidak correction in SPSS Version 20. p-values less than 0.05 after adjustment were considered statistically significant. Statistical analysis was performed using SAS 9.3.

A more detailed material and methods section has been provided as a supplement.
Results

LCN2 levels are elevated in MF plasma and MF MNC conditioned media.

PV, ET and PMF plasma LCN2 levels were significantly greater than normal control plasmas (p<0.0001)(Figure 1a). PMF plasmas contained significantly higher levels of LCN2 (median=93.8ng/ml, range: 6.9-329.0) than PV (median=54.8ng/ml, range: 2.7-268.0) or ET (median=47.4ng/ml, range: 13.3-167.7) plasmas (p=0.0175). Furthermore, plasma LCN2 levels from patients with PV-MF (median=160.7ng/ml, range: 9.9-281.1) as well as ET-MF (median=93.1ng/ml, range: 31.7-173.5) were significantly greater than that present in PV or ET plasmas, respectively, (p=0.0002 and p=0.0175, respectively)(Figure 1a). An inverse relationship between both patient hemoglobin levels (p<0.0001) (Figure 1b) and platelet counts and LCN2 levels was observed (p=0.0044)(Figure 1c). However, the plasma LCN2 levels were not significantly different in those patients that had more severe degrees of MF (Figure 1d). The levels of plasma LCN2 were not related to the degree of elevation of number of WBCs (Figure S1), the type of treatment received (Figure S2), patient age (Figure S3), gender (Figure S4) or JAK2V617F status (Figure S5).

LCN2 levels were significantly greater in MF than PV MNC conditioned medium(CM), MNCs or normal BM MNC (p=0.0034)(Figure 2a). MF T and B cell depleted PB MNC cells expressed higher levels of LCN2 than normal BM MNC depleted of T and B cells (Figure 2b). A greater proportion of MF than normal MNCs expressed LCN2 (Figure 2c). In addition, immunohistochemical analysis of PMF BM biopsy specimens revealed that LCN2 expression was restricted to cells belonging to the myeloid lineage rather than erythroid or megakaryocytic cells (Figure 2d).
BCR-ABL protein activates the expression of LCN2 and represses LCN2 receptor expression, rendering BCR-ABL+ cells refractory to secreted LCN2(18). This finding led us to examine LCN2 receptor expression by both normal BM and MF MNC and CD34+ cells. The percentage of MF MNC, CD34+ and CD34- cells expressing the LCN2 receptor was lower than that observed in normal BM (p=0.0081 and p=0.0122; Figure 2e-g). However, LCN2 receptor mRNA levels in MF CD34+ cells were similar to that observed in normal CD34+ cells (Figure 2h and 2i), suggesting that increased MF LCN2 receptor expression was a consequence of events that occurred post-translationally. In order to further examine if the downregulation of LCN receptor was the consequence of chronic exposure to LCN2, we incubated normal BM CD34+ cells from three different donors with LCN2 for varying periods of time (30 min to 3 days). We were unable to detect a significant reduction in the percentage of CD34+ cell expressing the LCN2 receptor (data not shown).

**LCN2 promotes the proliferation of MF hematopoietic cells but not normal BM and PB cells**

LCN2 increased PMF CD34+ cell numbers but decreased normal CD34+ cell numbers (p=0.007) (Figure 3a). Also, LCN2 decreased the numbers of assayable normal marrow CFU-GM–but not BFU-E (p=0.001 and p>0.05, respectively, Figure 3b and Table S1). By contrast, LCN2 did not inhibit but rather promoted MF BFU-E- and CFU-GM- derived colony formation (p=0.02 and p=0.009 respectively, Figure 3c and Table S2). Similarly, LCN2 treatment significantly decreased the numbers of CFU-GM-derived colonies generated from normal G-CSF mobilized peripheral blood CD34+ cells (p=0.03 and p=0.047, respectively, Figure S6 and Table S3). The proliferative effect of LCN2 on MF HPC was observed irrespective of the patient’s JAK2V617F status (data not shown). Exposure of CD34+ cells from patients with JAK2V617F positive MF to
LCN2 did not alter the overall percentage of JAK2V617F positive colonies, but increased the proportion of homozygous JAK2V617F positive colonies in 3 of 4 cases (Table 1).

**LCN2 induces DNA double-strain breaks and apoptosis of normal BM but not PMF MNC and CD34⁺ cells**

LCN2 significantly increased the proportion of normal BM but not MF CD34⁺γ-H2AX⁺ cells (p=0.007, Figure 4a and Table S4). We then assessed if reactive oxygen species (ROS) were responsible for the accumulation of DNA strand breaks by measuring the levels of intracellular ROS in normal and MF MNCs by loading cells with DCFDA. LCN2 treatment of normal but not PMF MNCs was associated with increased ROS activity (p=0.011)(Figure 4b). PMF MNC CM was capable of increasing the proportion of normal H2AX⁺ MNC to a similar degree as purified LCN2. The administration of an LCN2 antibody, furthermore, reduced the proportion of CD34⁺γ-H2AX⁺ generated in the presence of MF CM (p=0.001) (Figure 4c and 4d).

Exposure of normal BM CD34⁺ cells to LCN2 dramatically increased the number of AnnexinV⁺ cells in a dose-dependent fashion (Figure 4e and 4f). This effect was blocked by addition of N-Acetyl-Cysteine (NAC), a reactive oxygen species (ROS) scavenger. By contrast, LCN2 with or without NAC did not increase the numbers a MF CD34⁺AnnexinV⁺ cells (Figure 4g and 4h). These data indicate that LCN2 not only selectively promotes the survival of PMF CD34⁺ cells but also leads to the apoptosis of normal BM CD34⁺ cells by increasing ROS.

**LCN2 mediates bone marrow stromal cell proliferation by acting through the ROS pathway**
We next determined if the LCN2 receptor was expressed by normal MSCs. The LCN2 receptor was shown to be expressed by virtually all normal ACs (Figure 5a and 5b). We then monitored the effect of LCN2 on normal marrow AC (MAC). After three weeks of incubation, increased proliferation of MAC was observed in cultures containing LCN2 alone (Figure 5c and 5d). Cultures with LCN2 contained fewer non-AC but greater numbers of spindle shaped AC. Similar results were obtained with 6 different normal BM samples. In addition, the ability of LCN2 to promote MAC proliferation was blocked by addition of NAC (Figure 5e and 5f). These data suggest that LCN2 acts on normal MSCs to promote the generation of ROS which induces MAC proliferation. We then determined if LCN2 secreted from PMF MNCs similarly affected the proliferation of MAC. MNCs from normal BM were co-cultured with MF MNC samples known to elaborate LCN2 levels greater than 20 ng/ml/10^5 cells (Figure S6 a, b and c). The density of MAC was dramatically increased and appeared spindle shaped in wells containing LCN2 producing MF MNC cells (Figure S6 b). The density of normal BM MNC cells that remained in suspension was reduced (Figure S6 b). Similar changes were not observed following the culture of BM MNC with normal or MF BM MNC known to elaborate lower levels of LCN2 (Figure S6 b).

Phenotypic and functional analysis of LCN2 primed adherent cells and mesenchymal stem cells (MSC)

Normal MAC in both control cultures and cultures containing LCN2 were CD45^- and CD34^-, but expressed vimentin, α-smooth muscle actin (α-SMA), CD90, CD105 and CD106 (Figure 6a-e). These phenotypic properties are consistent with adherent stromal cells with phenotypic properties associated with fibroblasts and MSC(19).
We next determined if the incubation of normal MSCs with LCN2 altered their differentiation pattern. MSCs were pre-incubated with or without LCN2 for at least ten days in MSCGM, and then in media that favored osteocyte or adipocyte formation. The differentiation of MSCs towards osteoblast like cells was enhanced by prior treatment with LCN2 as compared to MSCs not pre-treated (Figure 6f and 6g). The fluorescence intensity of staining for osteocalcin, a protein maker of bone forming cells was significantly increased in MSC cells pre-treated with LCN2 (Figure 6h). By contrast, when LCN2 pre-treated cells were then cultured under differencing conditions that favored adipocyte formation, adipogenesis was clearly impaired by the addition of LCN2 (Figure 6i and 6j). The fluorescence intensity of staining for the fatty acid binding protein 4 (FABP4) was markedly decreased in LCN2 treated cells (Figure 6k). These data collectively indicate that LCN2 alters the differentiation pattern of normal MACs by promoting osteoblast and fibroblast but not adipocyte development.

The effects of LCN2 on MSC gene expression

As can be seen in Figure 7, after a single day of LCN2 exposure, the expression of runt-related transcription factor 2 (RUNX2), a master transcription factor associated with osteoblast differentiation (20-23) was significantly increased (Figure 7a). Similarly, the expression of TGF-β and VEGF, transcripts both growth factors known to be important in remodeling the MF microenvironment (24-26) were also increased (Figure 7b), while bone morphogenetic proteins 2 (BMP2), a member of the TGF-β super family involved in osteoblast differentiation (25-27) was increased on day one and day six of culture. In addition, two known products of osteoblasts, osteoprotegerin (OPG) and collagen type1(COL1A1) (20-21) were also increased after incubation with LCN2 (Figure 7c). By contrast, incubation with LCN2 markedly decreased PPARγ gene expression which is associated with adipogenesis (Figure 7d). In order to further
understand if the ROS pathway plays a role in LCN2 alterations in BM MSC differentiation, NAC was added to LCN2 treated MSCs. NAC not only inhibited \textit{RUNX2} gene expression induced by LCN2 (Figure 7f) but also inhibited \textit{TGF-\beta}, \textit{BMP2}, \textit{VEGF} and \textit{OPG} genes expression (Figure 7f). These data suggest that ROS is one of pathways by which LCN2 affects BM MSC differentiation. The pattern of gene expression illustrated in Figure 7e summarizes the pattern of genes that are expressed at different time points after incubation of MSCs.

\textbf{MF BM AC expression increased \textit{RUNX2} transcripts and are capable of responding to LCN2}

We then questioned whether BM MSC cells from MF patients had similar responses to the LCN2 as normal MSC. To address this question, the gene expression pattern of two individual MF BM AC cells was compared to that of normal marrow AC. Similarly to normal ACs, the MF AC cells expressed the LCN2 receptor (Figure 7g), and MF AC cells also expressed higher levels of \textit{RUNX2} but not \textit{TGF-\beta} and \textit{BMP2} transcripts as compared to normal BM ACs (Figure 7h). Importantly, after exposure to LCN2 for ten days, the expression of the \textit{RUNX2}, \textit{TGF-\beta}, \textit{OPG} and \textit{VEGF}, and \textit{COLIA1} gene expression was still increased, while \textit{BMP2} gene expression was modestly affected (Figure 7i). Similar to normal BM AC, incubation with LCN2 decreased the expression of the \textit{PPAR\gamma} (Figure7i). These data suggest that MF BM ACs are capable of responding to the effects of LCN2 by increasing the expression of several target genes.
Discussion

Several groups have reported that LCN2 gene expression is upregulated in MPNs and that plasma LCN2 levels are increased (10-13). In addition a growing number of studies have provided evidence that a variety of solid tumors are characterized by overexpression of LCN2 (28-31). Previously, LCN2 has been implicated in the pathobiology of another MPN, CML, where this iron containing protein has been shown not only to promote BCR/ABL+ hematopoiesis but also inhibiting the residual reservoir of normal hematopoietic progenitor cells, thereby likely contributing to the clonal dominance of the malignant clone (14, 18). Beyond its effects on tumor cells, LCN2 expression has also been implicated as a mediator of renal, pulmonary and cardiac fibrosis (32-34).

In this report, the levels of LCN2 in the plasmas of MF patients (PMF, PV-MF and ET-MF) were each shown to be greater than that present in normal control plasma; findings which extends the observations of others (10-13). Furthermore, the LCN2 levels present in the MF plasmas were 2-3 fold greater than that observed in patients with proliferative MPNs (PV and ET) providing a possible link between increased LCN2 levels and progression to MF. An inverse relationship between plasma LCN2 levels and Hgb levels and platelet counts but not WBC numbers in MPN patients was observed, suggesting that LCN2 might be related to the development of anemia and thrombocytopenia. The degree of increase in plasma LCN2 levels in patients was not related to gender, age, type of treatment received or the severity of MF. Importantly, MF MNC CM contained elevated LCN2 levels and LCN2 mRNA expression levels were higher in lymphocyte depleted MF MNC cells. LCN2 was localized to myeloid cells within MF marrow cells. Although LCN2 transcripts were documented in this report to be detectable in normal and MF CD34+ cells (12, 13), LCN2 synthesis has been previously shown to occur
almost exclusively in marrow myelocytes and metamyelocytes (35-38). The addition of either purified LCN2 or MF CM containing LCN2 induced DNA damage and apoptosis in normal CD34+ cells but not MF CD34+ cells and affected hematopoietic colony formation in a corresponding fashion. These findings differ from those reported by Kagoya and coworkers (11) who observed that LCN2 inhibited both normal as well as JAK2V617F positive hematopoiesis. These discrepancies can be attributed to cells from different species being studied or the phase of MPN examined in the two studies being different (PV versus MF). Barosi and co-workers observed in a longitudinal study of MF patients a consistent rate of transformation from a heterozygous to a homozygous JAK2V617F mutational status which was associated with disease progression (39). In the present report, the addition of LCN2 to MF CD34+ cells resulted in an increase in the proportion of hematopoietic colonies that were homozygous rather than heterozygous for JAK2V617 indicating that LCN2 favors the persistence of JAK2V617F homozygous rather than JAK2V617F heterozygous or JAK2 wild type progenitor cells.

The inhibitory effect of MF CM on normal hematopoiesis was observed to be independent of the JAK2V617 status of the MF MNC donor. Kagoya and co-workers (11) have reported that increased LCN2 levels were also present in MPN patient plasmas with calreticulin as well as JAK2 mutations. The expression of the LCN2 receptor by MF MNC and CD34+ cells was observed to be down-regulated, yet the levels of LCN2 receptor mRNA were similar in normal and MF CD34+ cells. These data suggested that down regulation of LCN2 receptor is a consequence of events that occurred post-translationally. Sheng and co-workers have, however, reported that in murine cell lines that BCR/ABL repressed the LCN2 receptor expression by inducing a switch in binding from RUNX3, an activator of LCN2 receptor expression, to RUNX1, a repressor of LCN2 receptor expression (40). Our findings are, however, similar to the
reduced expression of the thrombopoietin receptor that has been reported in the platelets of PV and MF patients, which was reported to not a consequence of Mpl gene disruption or transcriptional repression but rather associated with incomplete Mpl glycosylation (41-42). Furthermore, Pecquet et al have demonstrated that JAK2V617F is associated with an increase in Mpl degradation by the proteasome (43-44). Whether a similar mechanism could account for the reduced expression of the LCN2 receptor by MPN hematopoietic cells will require further study.

We demonstrated that LCN2 was able to damage normal but not MF progenitor cells by increasing the generation of ROS. Marty and coworkers have previously defined a role for ROS in JAK2V617F positive hematopoiesis (45). They demonstrated in a mouse model that JAK2V617F induced the accumulation of ROS, oxidation of DNA and accumulation of DNA strand breaks (45).

As MF progresses depletion of marrow hematopoietic cells is accompanied by progressive marrow fibrosis, increased marrow microvessel density and osteosclerosis (1, 46-48). MF associated osteosclerosis has been associated with increased numbers of osteoblasts and reduced numbers of osteoclasts (1, 49-50). The cells comprising MF marrow microenvironment are not directly affected by the malignant process but their differentiation patterns are influenced by cellular products elaborated by cells belonging to the MF clone(3). The coupling of the MF associated clonal myeloproliferation with characteristic microenvironmental changes has been attributed to the intramedullary release of growth factors by dysplastic megakaryocytes and monocytes that subsequently activate MSCs. In addition increased production of osteoprotegerin (OPG) by stromal cells has been implicated in the unbalanced osteoblast production which results in MF related osteosclerosis(49). We provide evidence that the sequence of events leading to abnormalities of hematopoiesis and the microenvironment in MF can be attributed at least in
part to LCN2 elaborated by malignant myeloid cells. We observed that MF MNC CM containing high concentrations of LCN2 as well as purified LCN2 were able to promote stromal cell proliferation. These effects were eliminated by the addition of NAC suggesting that the stromal cell proliferation was due to the generation of increased ROS. Furthermore, we demonstrated that LCN2 was capable of promoting osteoblast but not adipocyte differentiation of MSCs. The ability of LCN2 to increase osteoblastic differentiation is clearly reminiscent of the findings of Schepers and co-workers, who were able to show that malignant MPN myeloid cells elaborated a series of factors which stimulated murine MSCs to overproduce osteoblasts (51).

We have documented that LCN2 can create a cascade of events which fuel the development of the MF phenotype. The rapid upregulation of RUNX2 within MSCs with LCN2 treatment is likely a pivotal step in their commitment to osteoblasts (21-23). Furthermore, the up-regulation of TGF-β and BMP2 which follows the expression of RUNX2 likely further contributes to the amplification of the numbers of osteoblasts (26-27). In addition the expression of BMP2 and VEGF may play a role in the development of increased marrow micro-vessel density (23,27). The coupling of osteoblast precursors with vascular repair has been previously reported (52), indicating that osteoblastogenesis and angiogenesis are likely directionally steered by the release of stimulatory signals such as VEGF and BMP2. The increased expression of OPG by LCN2 primed MSCs likely contributes to the development of osteosclerosis in MF since OPG promotes additional bone formation by binding to RANKL thereby preventing osteoclasts formation(53). Additionally, the increased expression of transcripts for the extracellular matrix protein collagen type1 (COL1A1) by LCN2 stimulated MSCs is especially intriguing since type 1 collagen is increased within the marrows of patients with the most advanced forms of MF(1, 21). Consistent with these observations, MF marrow AC cells contained a higher degree of expression of
RUNX2 transcripts as compared to the normal MSCs which we speculate might be due to the priming of MF MSCs by LCN2 within the marrow microenvironment in man. Furthermore, the expression of the RUNX2, TGF-β, OPG and VEGF, and COL1A1 gene expression was increased while PPARγ transcripts were decreased in primary normal and MF marrow MSC treated in vitro with LCN2, suggesting that LCN2 might play a pivotal role in remodelling of the MF marrow microenvironment.

We demonstrated that normal as well as MF MSCs express the LCN2 receptor and that LCN2 affects MSCs by promoting the generation of ROS. ROS are well known to have diverse biological consequences which are tissue specific (54-57). ROS has also been implicated previously as a determinant of MSC behavior (58-61) in cancer patients. We document that a ROS inhibitor not only blocked BM MSC proliferation, but also reduced the expression of genes associated with osteoblastic differentiation. These data are somewhat surprising since ROS has previously been reported to reduce adherence and adipocyte differentiation of rat MSCs(62-63). These discrepancies can be attributed to either differences in the responses of rat and human MSCs to ROS or pathways in addition to ROS contributing to the effects of LCN2 on human MSC differentiation.

The data presented here are illustrative of the complex interplay between the MF malignant hematopoietic cells and the marrow microenvironment (Figure 7j) which ultimately leads to the establishment and possible evolution of the MF clinical phenotype.
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Authors Contributions:

M.L designed, performed the experiments, analyzed data and wrote the paper. L.X. Y.L. performed the experiments. L.B., D.A and J.L assisted the experiment. T.H and J.G. analyzed data, and participated in writing of the manuscript. R.W. banked the plasma specimens, provided samples. R.H. designed experiments, analyzed data and wrote the paper. All authors read and approved the manuscript.

Competing financial interests

All authors declare no competing financial interests.
References:


vera and contribute to the growth of clonal erythroblasts independently of JAK2V617F. *Oncogene.* 2011; 30(8): 990-1001.


Figure Legends

Figure 1. LCN2 levels are elevated in MPN plasma
(a) An enzyme-linked immunosorbent assay (ELISA) was used to quantitate LCN2 levels in plasma isolated from normal donors (n=16) and patients with PV (n=30), ET (n=30), PMF (n=30), PV-MF (n=20) and ET-MF (n=20), Kruskal-Wallis non parametric analysis of variance p<0.0001 comparing normal plasma versus plasma from each type of MPN, PMF versus PV or ET (p=0.0175); plasma LCN2 levels in PV-MF as well as ET-MF were greater than that present in PV or ET plasmas, respectively (Wilcoxon Rank Sum Tests, p=0.0002 and p=0.0175, respectively). The numbers indicate the median LCN2 level present in the plasma of patients with a particular MPN; (b) The non-parametric Spearman correlation coefficient analysis showed an inverse relationship between hemoglobin levels and LCN2 levels (coefficient= -0.35, p<0.0001, n=117); (c) and an inverse correlation between platelet counts and LCN2 levels (coefficient= -0.26, p=0.0044, n=117); (d) The plasma LCN2 levels were not significantly different in those patients that had more severe degrees of MF as assessed by the number of prognostic variables that characterized their MF, Kruskal-Wallis non parametric analysis of variance p>0.05 at number 0 vs each of others.

Figure 2. LCN2 levels are elevated in PMF MNC CM and LCN2 is localized to MF marrow myeloid cells
(a) LCN2 levels in media conditioned with MF and PV mononuclear cells (MNC) (n=10 and n=7, respectively) and normal bone marrow MNCs (n=10) after incubation in serum free media containing SCF, TPO, FL-3L and IL-3 for 1 day, Kruskal-Wallis non parametric analysis of variance p<0.0001 comparing Normal vs MF as well as PV versus MF; (b) Quantitative RT-PCR
results showed that LCN2 transcript levels were greater in MF T and B cell depleted MNC cells as well as in MF CD34+ cells, (repeated measures ANOVA F test p=0.02 and p=0.002, respectively, n=5); (c) Immunofluorescence staining with an anti-LCN2 antibody showing LCN2 (green) presented in normal and MF MNC, nuclei were stained with DAPI (original magnification x200). A greater proportion of MF MNC expressed LCN2 than normal MNC; (d) Photomicrograph demonstrating LCN2 expression by cells within a bone marrow biopsy specimen from an MF patient. LCN2 was expressed by myeloid cells but not by erythroid precursors or megakaryocytes (original magnification x400). (e and f) Flow cytometric analysis of LCN2 receptor expression by normal BM and MF MNCs and CD34+ cells; (g) A greater proportion of normal BM MNC (n=5) expressed the LCN2 receptor than MF MNCs (n=6) (Wilcoxon Rank Sum Test, p=0.008); (h) Similarly, a greater proportion of normal BM CD34+ cells (n=5) expressed the LCN2 receptor than MF CD34+ cells (n=5) (Wilcoxon Rank Sum Test, p=0.012); (i) A greater proportion of normal BM CD34- cells (n=5) expressed the LCN2 receptor than MF CD34- cells (n=5) (Wilcoxon Rank Sum Test, p=0.03); (j) qRT-PCR studies indicated that LCN2 receptor gene expression was similar in both MF and normal T and B cell depleted MNC cells samples; (k) qRT-PCR studies indicated that that LCN2 receptor gene expression was similar in both MF and normal CD34+ cells.

Figure 3. LCN2 promotes the proliferation of MF hematopoietic cells but suppresses normal BM cell proliferation

(a) The addition of increasing doses of LCN2 increased MF (n=7) but not normal BM CD34+ cell numbers (n=6, repeated measures ANOVA F test, adjusted for normal BM vs. MF **: p<0.01); (b) LCN2 reduced normal BM CFU-GM derived colony formation (repeated measures
ANOVA F test p=0.001 and p<0.05, n=6); (c) LCN2 promoted MF BFU-E derived colony formation, (repeated measures ANOVA F test p=0.02 and p=0.009 respectively, n=11).

Figure 4. LCN2 induces DNA damage and increases ROS in normal but not MF CD34+ cells

(a) Treatment with LCN2 increased the proportion of normal but not MF CD34+/γ-H2AX+ cells (repeated measures ANOVA F-test p=0.001, n=4); (b) Flow cytometric analysis of intensity of DCFDA fluorescence, used to quantitate intracellular ROS, in normal and MF CD34+ cells (n=5 and n=6, respectively); (c) Flow cytometric analysis showing that treatment with MF CM increased the proportion of normal-γ-H2AX+ BM MNC to a similar degree as that induced by 20 ng/ml of LCN2. Prior incubation of an LCN2 antibody (R & D Systems) with MF MNC CM reduced the proportion of γ-H2AX+ normal BM MNCs; (d) The increase in the proportion of normal γ-H2AX+ BM MNC following the addition of LCN2 was not observed with the addition of an LCN2 antibody (Repeated measures ANOVA F-test p=0.001, n=4); (e and f) LCN2 treatment of normal BM CD34+ cells dramatically increased the percentage of CD34+Annexin V+ cells, this increase was not observed with the addition of N-Acetyl-Cysteine (NAC) to LCN2 containing cultures (Repeated Measures ANOVA F-Test NAC p=0.0008 and LCN2 p=0.0081, n=8); (g and h) The percentage of MF annexin V+CD34+ cells was not increased following the addition of LCN2; or the addition of NAC to LCN2 containing cultures (repeated Measures ANOVA F-Test p>0.05, n=6).

Figure 5. LCN2 promotes the proliferation of BM stromal cells which express the LCN2 receptor

(a) Immunofluorescence staining using an anti-LCN2 receptor antibody (SLC22A17) indicating that normal BM ACs express the LCN2 receptor (green), the nuclei were stained with DAPI,
images represent the analysis of AC from two different donors. (b) Western blot analysis of 4 different samples of normal BM AC cells indicated the expression of the LCN2 receptor. (c) Phase contrast microscopy showing AC confluency that occurred after the culture of BM MNC with at 0, 10, 20 and 50 ng/ml of LCN2 (original magnification x200); (d) The degree of ACs proliferation was assessed with the PrestoBlue cell viability reagent; cell numbers were determined based upon the fluorescence intensity at 560 nm. LCN2 treatment led to the generation of greater numbers of AC by normal marrow MNCs (Repeated Measures ANOVA F test p=0.008, n=7); (e) Immunofluorescence analysis of vimentin expression showed that ACs proliferation was impaired by the addition of NAC to LCN2 containing cultures. Similar results were obtained with 3 additional BM MNC samples; NAC alone did not affect AC proliferation (f) Analysis of fluorescence intensity of vimentin expression showed a significantly increased expression in ACs from BM MNCs cultured with LCN2 alone (repeated measures ANOVA F test p=0.008, n=4), which was not observed with the addition of NAC alone or NAC plus LCN2.

**Figure 6. Phenotype of Adherent cells treated with LCN2 and lineage differentiation potential of LCN2 treated adherent cells**

Immuo-labeling of marrow AC cultured in the presence and absence of lipocalin The ACs cultured under each of these conditions expressed (a) vimentin (green); (b) α-SMA (red); (c) CD105 (red); (d) CD106 (green); (e) CD90 (red) and CD106 (green) (original magnification x200); (f) Immunostaining of BM ACs that were pre-incubated with MSC culture medium alone for ten days and then cultured with media that promoted osteogenesis for 21 days; (g) Immunostaining of BM ACs that had been pretreated with LCN2 in MSC culture medium and then cultured under osteoblast inducing conditions. Osteoblastic differentiation of MSCs was clearly enhanced by pre-incubation with LCN2 for ten days; an anti-osteocalcin antibody was
used to identify osteoblasts, the cell nucleus was stained with DAPI. (h) The fluorescence intensity of osteocalcin was significantly increased in BM AC pre-treated with LCN2 (repeated measures ANOVA F test p=0.0015, n=6). (i) Immunostaining showed a greater number of adipocytes in BM ACs-cultured in the MSC medium and then exposed to adipogenesis medium; (j) Immunostaining revealed that adipogenesis was diminished by pre-treatment of BM ACs with LCN2 for ten days and then cultured in adipogenesis media. Anti-FABP4 antibody was used to identify adipocytes, cell nuclei were stained with DAPI; (k) Fluorescence intensity analysis revealed significantly decreased FABP4 protein expression in BM AC pre-treated with LCN2 (repeated measures ANOVA F test p<0.0001, n=6).

**Figure 7. LCN2 treatment of BM ACs induces expression of genes associated with osteoblastic but not the adipocytic differentiation program**

Quantitative RT-PCR revealed that; (a) *RUNX2* transcript levels were increased one day after incubation with LCN2; (b) *VEGF*, *TGF-β* and *BMP2* transcripts were also increased by the addition of LCN2 in a dose dependent fashion after one day; (c) LCN2 treatment increased *OPG* and *COLIA1* gene expression after ten days of incubation; (d) *PPARγ* gene levels were decreased after one day of incubation with LCN2; (e) 3-D area graph showing the time course of expression of various genes by BM ACs after incubation with LCN2 (50 ng/ml); (f) Addition of NAC blocked the expression of *RUNX2*, *VEGF*, *TGF-β*, *BMP2* and *OPG* transcripts induced by LCN2 after one day; (g) Immunofluorescence staining with an anti-LCN2 receptor antibody (SLC22A17) revealed that MF BM AC cells expressed the LCN2 receptor (green), the nuclei were stained with DAPI. (h) qRT-PCR results revealed that *RUNX2* transcription levels were increased in untreated MF BM AC cells; (i) qRT-PCR showed that ten days of treatment with
LCN2 increased *RUNX2, TGF-β, VEGF,* and *OPG* gene expression but decreased *PPARγ* gene expression by MF BM AC; (j) Cartoon summarizing proposed effects of LCN2 on hematopoietic progenitor cells and BM stromal cells in MF.
Table 1. Effect of LCN2 on the genotype of MF hematopoietic colonies

<table>
<thead>
<tr>
<th>Case</th>
<th>JAK2 Genotype of Hematopoietic Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Homo*</td>
</tr>
<tr>
<td>MF 1</td>
<td>0 (0)***</td>
</tr>
<tr>
<td>MF 2</td>
<td>10 (43.5)</td>
</tr>
<tr>
<td>MF 3</td>
<td>11 (52)</td>
</tr>
<tr>
<td>MF 4</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

1, 24 colonies were randomly selected for each condition; *Homo: Homozygous; ** Hete: Heterozygous. *** Raw data (% of total)
Figure 1
Figure 2
Figure 3

(a) Number of CD34+ cells (% of control) vs. LCN2 (ng/ml)

(b) Number of colonies (% of control) vs. LCN2 (ng/ml)

(c) Number of colonies (% of control) vs. LCN2 (ng/ml)
Figure 4
Lipocalin produced by myelofibrosis cells affects the fate of both hematopoietic and marrow microenvironmental cells

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