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Activation of non-canonical TGF- β 1 signaling indicates an autoimmune mechanism for bone marrow fibrosis in primary myelofibrosis

Fiorella Ciaffoni¹, Elena Cassella², Lilian Varricchio², Margherita Massa³, Giovanni Barosi^{3,4}, and Anna Rita Migliaccio^{1,2,4}

¹Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy

²Tisch Cancer Institute, Mount Sinai School of Medicine, New York, NY, USA

³Biotechnology Research Area, Center for the Study of Myelofibrosis, IRCCS Policlinico San Matteo Foundation, Pavia, Italy

⁴Myeloproliferative Disease Research Consortium, New York, NY, USA

Abstract

Primary myelofibrosis (PMF) is characterized by megakaryocyte hyperplasia, dysplasia and death with progressive reticulin/collagen fibrosis in marrow and hematopoiesis in extramedullary sites. The mechanism of fibrosis was investigated by comparing TGF- β 1 signaling of marrow and spleen of patients with PMF and of non-diseased individuals. Expression of 39 (23 up-regulated and 16 down-regulated) and 38 (8 up-regulated and 30 down-regulated) TGF- β 1 signaling genes was altered in marrow and spleen of PMF patients, respectively. Abnormalities included genes of TGF- β 1 signaling, cell cycling and abnormal in chronic myeloid leukemia (*EVII* and *p21^{CIP}*) (both marrow and spleen) and Hedgehog (marrow only) and p53 (spleen only) signaling. Pathway analyses of these alterations predict increased osteoblast differentiation, ineffective hematopoiesis and fibrosis driven by non-canonical TGF- β 1 signaling in marrow and increased proliferation and defective DNA repair in spleen. Since activation of non-canonical TGF- β 1 signaling is associated with fibrosis in autoimmune diseases, the hypothesis that fibrosis in PMF results from an autoimmune process triggered by dead megakaryocytes was tested by determining that PMF patients expressed plasma levels of mitochondrial DNA and anti-mitochondrial antibodies greater

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Corresponding author: Anna Rita Migliaccio, PhD, Department of Medicine, Mount Sinai School of Medicine, One Gustave L Levy Place, box #1079, New York, NY 10029, USA. Phone no: 212-2416974; Fax no: 212-8765276; annarita.migliaccio@mssm.edu.

AUTHORSHIP AND CONFLICT OF INTEREST STATEMENTS

Fiorella Ciaffoni, Elena Cassella and Margherita Massa performed experiments and analyzed data.

Giovanni Barosi provided samples from PMF patients and normal donors and assured compliance of the study with institutional IRB.

Anna Rita Migliaccio, Lilian Varricchio and Giovanni Barosi designed research, analyzed the data and wrote the manuscript.

All the authors have read the manuscript, concur with its content and state that its content has not been submitted elsewhere.

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than normal controls. These data identify autoimmunity as a possible cause of marrow fibrosis in PMF.

Keywords

TGF- β 1; myelofibrosis; autoimmunity; inflammation

INTRODUCTION

Fibrosis is the pathological manifestation of end-stage organ failure in numerous clinical conditions ranging from systemic sclerosis, myocardial infarction, liver cirrhosis, cystic fibrosis to idiopathic pulmonary fibrosis (reviewed in ¹). To identify possible treatments for these diseases, studies in animal models and primary tissues have been performed to define the mechanism(s) that determines occurrence of fibrosis in different organs. It is now recognized that fibrosis results from deregulation of the normal process of wound healing driven by TGF- β which alters the equilibrium between biosynthesis of the extracellular matrix by activated fibroblasts and its degradation by scavenger cells.

In inflammation, fibrosis results from fibroblasts activated by TGF- β over-expression through canonical SMAD signaling (reviewed in ^{1,2} and Table S1). The expression signatures of organs affected by inflammation-related fibrosis include increased expression of TGF- β and increased activity of its downstream elements *SMAD2/3* which control the transcription of *COL1A1* and *COL1A2*, the genes encoding for the pro- α 1 and pro- α 2 chains of collagen type I. These signatures also include down-regulation of *BMP7*, the TGF- β family member that antagonizes TGF- β , and of its down-stream partners *SMAD2/SMAD7* (transcriptional repressors) and p53³⁻⁵. In human immunodeficiency virus (HIV) transgenic mice, an animal model for kidney fibrosis, inflammation induces an oxidative stress that increases the content of homeo-domain interacting protein 2 (*HIPK2*) which in turn activates TGF- β expression and canonical TGF- β signaling⁶.

By contrast, in animal models and patients with fibrosis associated with systemic sclerosis, a genetic autosomal dominant form of autoimmune scleroderma, fibroblasts are activated by ERK and/or p38, two members of the non-canonical MAPK TGF- β signaling^{7,8} (Table S1). Although the levels of active TGF- β in a transgenic mouse model carrying the scleroderma-specific fibrillin-1 mutation (*Fbn1*) are normal, treatment of these mice with either neutralizing TGF- β antibodies or inhibitors of ERK signaling reduced the presence of autoimmune biomarkers (antinuclear and anti-topoisomerase I antibodies) in blood and prevented skin fibrosis⁷. Additional genes of the non-canonical TGF- β signaling, or that inhibit canonical TGF- β signaling, that have been described over-expressed in fibrotic organs of patients affected by autoimmune diseases are *JUN/JUNB*, *GADD45B*, *EVII*, *STAT1* and *IL-6* (see Table S1).

Primary myelofibrosis (PMF), the most severe of the Philadelphia-negative myeloproliferative neoplasms, is characterized by hematopoietic failure, fibrosis and osteosclerosis in bone marrow (BM) and hematopoiesis in extramedullary sites⁹ and is associated with megakaryocyte (MK) hyperplasia, dysplasia and death by a pathological

process of neutrophil emperipoiesis¹⁰. Whether in PMF TGF- β 1 is implicated in BM fibrosis is still debated. BM from these patients contain increased levels of pro-inflammatory cytokines, including TNF α ¹¹ while those of total and bioactive TGF- β 1 are only 0.5-two-fold superior to normal^{12,13}. However, ablation of TGF- β 1 cures animal models induced by gain-of-function of thrombopoietin (TPO^{high} mice) or loss-of function of Gata1 (Gata1^{low} mice), respectively the growth factor and the transcription factor that control MK maturation^{13,14}. TPO^{high} mice express high levels of TGF- β 1¹⁴. As Fbn1-mice⁷, Gata1^{low} mice express normal levels of TGF- β 1 but express distinctive TGF- β 1 signaling abnormalities in BM and spleen that are rescued by treatment with the TGF- β 1 inhibitor¹³.

To clarify the role of TGF- β 1 in determining fibrosis in PMF, TGF- β 1 signaling of BM and spleen from the patients was profiled using an array similar to that previously investigated to study Gata1^{low} mice. This profile identified that the TGF- β 1 signaling signature of BM and spleen of PMF patients is clearly abnormal, confirming a role for this growth factor in the pathogenesis of this disease. The altered signature of BM indicated activation of non-canonical TGF- β signaling, suggesting the existence in these patients of an underlying autoimmune process. This hypothesis was tested by determining that the plasma of PMF patients contain levels of cell-free mitochondrial DNA and anti-mitochondrial antibodies greater than normal. The recognition that in PMF fibrosis may be associated with activation of non-canonical TGF- β signaling identifies novel possible pharmaceutical targets for this disease.

MATERIAL AND METHODS

Human Subjects

Signalling profiling was performed on mononuclear cells obtained from BM (n=3) and spleen (n=6) of patients, normal BM (n=3) (ABM008F-BM3366, ABM008F-BM3527 and ABM008F-BM3600, ALLCELLS, Emeryville, CA) and spleen (n=3) from males (<30-years old) who underwent splenectomy following trauma. BM was obtained from *JAK2V617F* patients while spleen was obtained from four *JAK2V617F*-mutated, one *MPL*-mutated and one triple negative *JAK2V617F*, *CALR* and *MPL* patient (Table S2). Cell-free DNA and auto-antibodies were determined on blood from 40 PMF patients within 0–6 months from diagnosis (Table S3). Inclusion criteria for this study were to be consecutive patients not treated with disease modifying agents such as chemotherapy, immunomodulators or JAK2 inhibitors. Seven out of the 40 patients analyzed had received hydroxyurea. Blood from non-diseased de-identified individuals (n=18–30) and essential thrombocythemia [ET, n=12, 8 patients from the Center for the Study of Myelofibrosis of IRCCS Policlinico S. Matteo, Pavia, Italy (Table S3) and 4 patients from the Tissue Bank of the Myeloproliferative Disease Consortium] as control. Diagnosis was established according to WHO criteria^{15,16}. All the samples were collected according to guidelines established by local ethical committees for human subject studies in accordance with the 1975 Helsinki Declaration revised in 2000.

TGF- β pathway profiling

RNA was prepared from mononuclear cells lysed in Trizol (Gibco BRL, Paisley, UK). Reverse transcription was performed with the RT² First Strand kit (SABiosciences, Frederick, MD). cDNA was dissolved in the RT² SYBR ROX qPCR Mastermix and profiled with the Human TGF- β RT² ProfilerTM PCR array (SABiosciences) using *glyceraldehyde-3-phosphate dehydrogenase (G3PDH)* as housekeeping control, as described by the manufacturer. Amplification reactions were performed with the ABI 7300 Real Time PCR System (SDS, Applied Biosystems, Carlsbad, CA). Biological consequences of gene expression alterations were predicted with the David Bioinformatic Database (David Bioinformatics Resources 6.7 NIAID/NIH). In selected cases, results obtained by microarray were validated by quantitative RT-PCR using primers specific for each gene (SABiosciences). Cycle thresholds (Ct) were calculated with the SDS software and expressed as 2^{-Ct} using *G3PDH* as calibrator. Fold changes were calculated as average 2^{-Ct} of the gene in the tested population/average 2^{-Ct} of the same gene in non-diseased controls and used to calculate values of fold regulation using the SABioscience program.

Cell-free mitochondrial (mDNA) and nuclear (nDNA) DNA determinations in plasma

Cell-free DNA was isolated from 1 mL of plasma using a QIAamp Blood DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions, dissolved in SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and amplified [10 ng/reaction] by real-time PCR using primers specific for human cytochrome *c* oxidase subunit III (forward 5'-ATGACCCACCAATCACATGC-3' and reverse 5'-ATCACATGGCTAGGCCGGAG-3')(mDNA) or human α -globin (forward 5'-CTC TTC TGG TCC CCA CAG ACT-3' and reverse 5'-GGC CTT GAC GTT GGT CTT G-3')(nDNA). Plasma levels of mDNA and nDNA were expressed in arbitrary units by multiplying reverse Cts per amount of cell-free DNA recovered per mL of plasma.

Determinations of anti-mitochondrial (AMA) and anti-nuclear (ANA) antibodies in plasma

Plasma levels of AMA were quantified with a kit that detects auto-antibodies against mitochondrial proteins for diagnosis of autoimmune liver cirrhosis¹⁷ (cat. No. MBS260123, MyBioSource, San Diego, CA). Plasma levels of ANA were assessed with a semi-quantitative kit that determines presence of antibodies against DNA fragments and intracellular nuclear proteins for diagnosis of autoimmune systemic sclerosis¹⁸ (cat. No. 3205, Alpha Diagnostic Int., San Antonio, TX).

Statistical methods

Results were expressed as mean ($\pm$ SD) and analyzed with Anova using Origin 6.0 for Windows (Microcal Software, Inc., Northampton, MA). Values among groups were considered significantly different with a p value < 0.05. TGF- β pathway profilings were analysed with the RT²ProfilerTM PCR Array Data Analysis (SABioscience). Genes were considered differentially expressed when the mean fold regulation was two-fold with a p value < 0.05 (2-tailed, Student's t-test).

RESULTS

Non-canonical MAPK-dependent TGF- β signaling is activated in BM from PMF patients

Microarray profiling identified abnormal expression of several TGF- β signaling genes in BM of PMF patients (Figure 1 and Table 1). Twenty-three genes were up-regulated by two-to-seventeen-fold and 16 down-regulated by two-to-ten-fold. Of those, two were significantly up-regulated (*GDF3* and *p21^{CIP1}*) while three were significantly down-regulated (*COL1A1*, *LEFTY1* and *TGFBR1*).

In the spleen of PMF patients, 8 genes were up-regulated and 30 down-regulated. Only one gene (*TGFB11*) was significantly up-regulated while 13 genes (*BMP2*, *BMPR2*, *COL1A1*, *ID2*, *IFRD1*, *IGF1*, *IL-6*, *p21^{CIP1}*, *SMAD7*, *TGFB2*, *TGFB1*, *TGFBR1* and *THBS1*) were significantly down-regulated (Table 1). Of note, the analysis of microarray data restricted to spleen of the three *JAK2V617F*-mutated patients gave results super-imposable to those obtained including data from both *JAK2V617F*-mutated and *JAK2V617F*-non mutated patients (compare Table 1 and Table S4).

The majority of the abnormalities observed were unique either for BM (25 out of 39 genes) or spleen (25 out of 31 genes) (Table 1 and Figure S1). Only 10 genes were altered in the same direction in both organs: four up-regulated (*FST*, *EVII*, *LTBP1* and *TGFB11*) and six down-regulated (*BMP2*, *BMP3*, *COL1A1*, *NODAL*, *TGFBR1* and *TGFB2*). Of those, only *COL1A1* was significantly down-regulated in both organs. Three genes were up-regulated in BM and down-regulated in spleen (*p21^{CIP1}*, *GADD45B* and *ID1*) and one down-regulated in BM and up-regulated in spleen (*LEFTY*). Alterations were statistically significant only for *p21^{CIP1}*, a gene encoding a protein downstream to the RAC pathway that inhibits apoptosis¹⁹ and expressed at low levels in stem/progenitor cells from Gata1^{low} mice²⁰.

The alteration signatures of BM and spleen of PMF patients lacked markers of canonical TGF- β signaling (such as up-regulation of *COL1A1* and *HIPK2* and down-regulation of *BMT7* and *STAT1*) associated with fibrosis related to inflammation. By contrast, it contained abnormalities suggesting activation of the non-canonical TGF- β signaling observed in fibrotic tissues of autoimmune disorders (up-regulation of *JUN/JUNB*, *EVII* and *STAT1*) (Tables 1 and S1). These indications were validated by comparing the expression of selected genes involved in non-canonical (*JUN*, *GADD45B*, *EVII* and *STAT1*) and canonical (*BMP4*, *BMP7*, *SMAD7* and *COL1A1*) TGF- β signaling by quantitative RT-PCR (Figures 2 and S1). This analysis also indicated that the up-regulation of *GADD45B* observed in the spleen of PMF patients is statistically significant.

Additional phenotypes of PMF patients predicted by the abnormal TGF- β signaling signatures of their BM and spleen

David pathway analyses of all genes that were abnormally expressed in BM of patients with PMF identified alterations in TGF- β , Hedgehog, and MAPK signaling and in genes that control cell cycle or are activated in chronic myelogenous leukemia (CML) and solid cancers (Table 2). Abnormalities observed in canonical SMAD-dependent TGF- β pathway predict increased osteoblast differentiation, G1 arrest and apoptosis, and reduced ubiquitin-mediated proteolysis, a pathway indispensable for erythroid maturation²¹. Abnormalities

observed in non-canonical MAPK-dependent TGF- β pathway predict increased apoptosis and fibrosis and include up-regulation of *EVII*, a gene whose over-expression in stem/progenitor cells induces loss of response to erythropoietin and impairs erythroid differentiation in mice^{22,23}. Predictions of increased G1 arrest and apoptosis are consistent with the hematopoietic failure observed in BM of PMF patients. In addition, the combination of reduced ubiquitin-mediated proteolysis and increased expression of *EVII* may explain why in PMF BM activation of the stress pathway predicted by the observed up-regulation of *BMP4*²⁴ is not sufficient to increase erythropoiesis at levels sufficient to compensate anemia. Over-expression of *EVII* in mice enhances megakaryocyte proliferation and in humans is associated with hematopoietic disorders characterized by dysmegakaryopoiesis²². Therefore, over-expression of *EVII* is also consistent with the increased megakaryopoiesis observed in BM of PMF patients and suggests that *EVII* may be the element downstream of the activated p38-MAPK signaling that controls megakaryopoiesis in primary myelofibrosis²⁵. Increased G1 arrest and apoptosis in BM are also predicted by alterations observed in genes controlling cell cycle or involved in CML (Table 2).

By contrast, David pathway analyses of all genes abnormally expressed in spleen did not consistently predict G1 arrest and apoptosis. Abnormalities observed in canonical TGF- β signaling and in genes associated with CML (*EVII* up-regulation) and those observed in non-canonical TGF- β and p53 signaling and in cell cycle control predict, respectively, increased and reduced levels of G1 arrest and apoptosis. It is possible that increased G1 arrest and apoptosis reflect the fate of hematopoietic precursors, which fail to mature in the spleen while reduced G1 arrest and apoptosis reflect the great stem/progenitor cells amplification occurring in this organ²⁶. The hypothesis that the spleen microenvironment may favor amplification of the PMF stem cell clone but not that of its hematopoietic precursors is also supported by a comparison of alterations of *NODAL* and *LEFTY*, two members of the TGF- β superfamily, observed in BM and spleen. *NODAL* cooperates with fibroblast growth factor to maintain embryonic stem cells in a pluripotent state²⁷ while *LEFTY* antagonizes *NODAL* by inhibiting proliferation and inducing apoptosis²⁸. Expression of *NODAL* and *LEFTY* were both down-regulated in BM, in agreement with the poor hematopoietic supportive ability of this microenvironment, but *NODAL* was down-regulated and *LEFTY* up-regulated in spleen, consistent with the idea that the spleen microenvironment promotes generation of hematopoietic progenitor cells from the stem cell compartment (down-regulation of *NODAL*) but then inhibits their maturation by inducing apoptosis (up-regulation of *LEFTY*). The poor erythroid maturation support provided by spleen of PMF patients is also suggested by the observation that, in spite of anemia, *BMP4* was not up-regulated and *HIPK2* (homeodomain-interacting protein kinase 2) a gene the deletion of which inhibits terminal erythroid maturation in mice²⁹, was down-regulated in this organ. By contrast, under-expression of *THBS1* (thrombospondin 1, a suppressor of megakaryopoiesis also known as *TSP*³⁰) may explain the great number of MK present in spleen of PMF patients.

Of particular interest are the different alterations in *GADD45B* expression observed in BM, up-regulated, and spleen, down-regulated (Table 1 and Figure 2). Since *GADD45B* promotes

repair and damage prevention of DNA in response to stress³¹, its down-regulation only in spleen may explain genomic instability and chromosomal abnormality accumulation occurring in hematopoietic stem cells in this organ³².

The plasma from PMF patients contains increased levels of mDNA and AMA

In cystic fibrosis, neutrophils presenting extracellular traps (NET) of autologous DNA released by cells dying for the autoimmune reaction are responsible for fibroblast activation and fibrosis³³. Morphological evidence exists in support of the hypothesis that NET may be generated also in PMF. In fact, BM of PMF patients contain numerous neutrophils engaged in a process of pathological emperipolesis with MK that results in death of MK by para-apoptosis^{10,34}. It is possible that during this process, neutrophils may form NET of mitochondrial and/or nuclear DNA released by the dead MK triggering fibrosis. To test this hypothesis, levels of mDNA and nDNA and AMA and ANA in plasma of a cohort of PMF patients (n=39–40) were compared to those present in plasma of ET patients (n=8–12) and non-diseased controls (n=18–30) (Figure 3).

Plasma from PMF patients contained levels of both mDNA and AMA significantly greater by 1.5–2-fold than that of controls. By contrast, nDNA was detected only in 1 out of 7 PMF patients analyzed and ANA was present at levels not statistically different from those observed in controls (Figure 3). Failure to detect nDNA and ANA in plasma from PMF patients may reflect the fact that the para-apoptotic process leaves intact the nuclear membrane³⁵ preventing exposure of genomic DNA and intranuclear proteins to immune cells.

DISCUSSION

The data described here indicate that, in spite the nearly normal levels of TGF- β detected in PMF patient so far^{12,13}, the TGF- β signaling profiling of BM and spleen of these patients is clearly altered. David-assisted pathway analyses identified similarities but also important differences in the biological consequences of the TGF- β signaling signatures of BM and spleen from these patients. Alterations of canonical TGF- β signaling predicted increased G1 arrest and apoptosis in both organs but increased osteoblast differentiation in BM only. Alterations in non-canonical TGF- β signaling predicted increased G1 arrest/apoptosis and fibrosis in BM and reduced DNA repair and damage prevention in spleen.

By inductive reasoning that generalizes the concept that non-canonical TGF- β signaling mediates fibrosis in autoimmune diseases, we hypothesized that in PMF BM fibrosis is an autoimmune manifestation triggered by NET formed during the process of pathological MK/neutrophil emperipolesis (Figure 4). This hypothesis was tested by demonstrating that the plasma of PMF patients contains levels of mDNA and AMA greater than normal. These results confirm a previous hypothesis for the presence of autoimmune dysfunction in PMF³⁶ based on reports indicating that plasma of PMF patients contains antibodies against red cells and platelets³⁷ and that the risk to develop myeloproliferative neoplasms is significantly increased by a prior history of autoimmune diseases³⁸.

In spite of some differences related to species-specific differences in organ functions, striking similarities were observed in the signature of TGF- β signaling abnormalities of BM and spleen in PMF patients and in the Gata1^{low} mouse model (Figure S3). The majority of the signature abnormalities common to BM of PMF patients and Gata1^{low} mice, including increased expression of two of the markers for autoimmune fibrosis (*STAT1* and *IL-6*) were normalized in the animal model by treatment with the TGF- β inhibitor that rescued its myelofibrotic phenotype¹³. This observation suggests that treatment with TGF- β inhibitors may also cure PMF patients. This hypothesis was partially confirmed by a recent clinical trial with GC1008, a human immunoglobulin G4 kappa monoclonal antibody that neutralizes each of the mammalian isoforms of TGF- β ³⁹ that provided evidence for reduced fibrosis in at least one of the three patients treated. This antibody is no longer available for investigation in PMF. However, the evidence that the non-canonical MAPK-driven TGF- β signaling is responsible for fibrosis in PMF provided here suggests that this antibody may be replaced by interferon- α , a compound that targets the p38-MAPK pathway^{40,41} currently undergoing clinical investigation in PMF, or by anyone of the eight ERK inhibitors and five p38 inhibitors already approved by FDA for clinical use in other cancers^{42,43}.

CONCLUSION

TGF- β signaling profiling provides evidence that BM fibrosis characterizing PMF patients is the consequence of activation of non-canonical MAPK-driven TGF- β signaling pathway. Since by contrast with SMAD signaling, inhibitors of non-canonical MAPK-driven TGF- β signaling are already used in the clinic, the expression profiling described in this study paves the way for future investigations that may lead to the identification of novel approaches for treatment of this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The bone marrow and spleen of PMF patients present unique alteration signatures of TGF- β signalling

Hierarchical clustering of normalized gene expression in bone marrow and spleen from non-diseased volunteers (controls, C, n=3) and PMF patients. Results were obtained with BM from three *JAK2V617F*-mutated PMF patients and spleen from four *JAK2V617F*-mutated (in black) and two *JAK2V617F*-mutated PMF patients.

Figure 2. Quantitative RT-PCR validation that the alteration signatures of bone marrow and spleen of PMF patients predict activation of non-canonical (up-regulation of *JUN*, *GADD45B*, *EVI1* and *STAT1*) rather than canonical (down-regulation of *BMP4*, *BMP7*, *SMAD7*, *STAT1*, and up-regulation of *COL1A1*), TGF- β signaling

Results are expressed in relative units considering the expression in the corresponding organs from non diseased volunteers (Ctrl) as 1 and are presented as mean ($\pm$ SD) of values observed with BM from three PMF patients and three non-diseased individuals and with spleen from five PMF patients and three non-diseased individuals (the same samples analyzed in Figure 1). To allow appreciation of the difference in gene expression between BM and spleen, Figure S1 presents results from both BM and spleen expressed in relative units considering values in BM from non diseased volunteers as 1.

Figure 3. Plasma from PMF patients contains levels greater than normal of cell-free mitochondrial DNA (A) and anti-mitochondrial antibodies (B) and normal levels of antinuclear antibodies (C)

Results are expressed as individual values (symbols) and as mean ($\pm$ SD) of values (columns) from non-diseased volunteers (Ctrl), essential thrombocythemia (ET) and primary myelofibrosis (PMF) patients. The same individuals were analyzed in A–C. The numbers of individuals included in each cohort is indicated in parenthesis. The yellow area in C indicates the range of optical density observed in the standards provided by the Kit as negative controls. See Material and Methods for further details.

Figure 4. A model for establishment of the autoimmune process possibly leading to fibrosis in PMF

Neutrophils presenting extracellular traps (NET) of autologous DNA trigger autoimmune reactions resulting in fibrosis in cystic fibrosis (Reviewed in ref 33). Using this mechanism as a foundation, we propose here that NET formed by mitochondrial DNA released by MK dying of para-apoptosis during the process of pathological emperipolesis with neutrophils (refs 10, 34, 35) may trigger the autoimmune reaction leading to fibrosis in PMF. Legend: Neu = neutrophil; MK = megakaryocyte; nu = para-apoptotic nucleus; mDNA = mitochondrial DNA.

Table 1

Fold regulation and p-value of genes differentially expressed in PMF patients with respect to non-diseased controls. Differences statistically significant are in bold.

BM	Over Expressed		Under Expressed	
Gene	Fold Reg.	p-values	Fold Reg.	p-values
<i>AMHR2</i>	5.4264	0.137		
<i>BGLAP</i>			-2.2397	0.392
<i>BMP2</i>			-4.6697	0.104
<i>BMP3</i>			-8.5544	0.215
<i>BMP4</i>	5.4264	0.137		
<i>BMP7</i>	6.8369	0.053		
<i>BMPRIA</i>			-2.2921	0.238
<i>CHRD</i>	5.4264	0.137		
<i>COL1A1</i>			-10.0794	0.022
<i>COL1A2</i>			-7.2267	0.556
<i>DLX2</i>	7.4643	0.071		
<i>ENG</i>			-2.0753	0.174
<i>EVII</i>	2.9828	0.371		
<i>FST</i>	3.6723	0.138		
<i>GADD45B</i> *	3.6638	0.117		
<i>GDF2</i>	5.4264	0.137		
<i>GDF3</i>	8	0.027		
<i>GDF5</i>	3.1529	0.16		
<i>GDF6</i>	5.4264	0.137		
<i>GSC</i>	5.4264	0.137		
<i>ID1</i>	17.1484	0.08		
<i>IL6</i>	15.9631	0.328		
<i>INHHA</i>	5.2416	0.128		
<i>INHBA</i>			-7.0453	0.109
<i>INHBB</i>	5.4264	0.137		
<i>JUN</i>	2.5847	0.084		
<i>LEFTY1</i>			-3.6301	0.00003
<i>LTBP1</i>	2.1535	0.176		
<i>LTBP2</i>	2.114	0.771		
<i>NODAL</i>			-2.3457	0.167
<i>NOG</i>			-5.3641	0.129
<i>P21CIP1 (CDKN1A)</i>	6.9003	0.034		
<i>P27KIP1</i> *			-2.5669	0.07
<i>SMAD2</i>			-2.7638	0.078

BM	Over Expressed		Under Expressed	
Gene	Fold Reg.	p-values	Fold Reg.	p-values
<i>STAT1</i>	2.0801	0.222		
<i>TGFB111</i>	2.4623	0.291		
<i>TGFB3</i>			-3.9541	0.084
<i>TGFBRI</i>			-2.1337	0.046
<i>TGFB2</i>			-2.783	0.092

Spleen	Over Expressed		Under Expressed	
Gene	Fold Reg.	p-values	Fold Reg.	p-values
<i>ACVR2A</i>			-2.0139	0.1822
<i>ACVRL1</i> *			-2.514	0.1304
<i>BAMBI</i>			-2.0946	0.5669
<i>BMP1</i>	2.308	0.1189		
<i>BMP2</i>			-2.7069	0.0364
<i>BMPER</i>			-2.3214	0.1458
<i>BMPRI1B</i>	4.7076	0.2322		
<i>BMPR2</i>			-2.4938	0.0036
<i>COL1A1</i>			-5.9107	0.0321
<i>EMPI</i> *			-8.1587	0.1945
<i>EVII</i>	20.3694	0.1139		
<i>FOS</i>			-2.5907	0.0597
<i>FST</i>	4.1506	0.2393		
<i>GADD45B</i> *			-2.4967	0.0612
<i>GDF3</i>	2.1861	0.3304		
<i>HERPUDI</i> *			-2.783	0.0785
<i>HIPK2</i> *			-2.0849	0.2538
<i>ID2</i>			-2.2553	0.0286
<i>IFRD1</i> *			-2.5817	0.0028
<i>IGF1</i>			-3.9908	0.0329
<i>IL6</i>			-3.9404	0.0071
<i>JUNB</i>			-2.1361	0.074
<i>LEFTY1</i>	2.9519	0.1136		
<i>LTBP1</i>	20.2054	0.2331		
<i>NODAL</i>			-2.4481	0.3805
P15INK4B (CDKN2B)			-2.3376	0.08
P21CIP1 (CDKN1A)			-3.1131	0.0116
<i>SMAD3</i>			-3.5105	0.0606
<i>SMAD7</i> *			-11.043	0.0379
<i>SMURF1</i>			-2.3054	0.1731

Spleen	Over Expressed		Under Expressed	
Gene	Fold Reg.	p-values	Fold Reg.	p-values
<i>TGFBIII</i>	6.5206	0.0145		
<i>TGFB2</i>			-4.8906	0.0272
<i>TGFB1</i>			-8.9693	0.0001
<i>TGFBRI</i>			-3.8415	0.0109
<i>TGFB2</i>			-2.8646	0.3092
<i>TGFB3</i>			-2.8547	0.7777
<i>TGFI</i> *			-2.0777	0.3115
<i>THBS1</i> *			-10.01	0.0048

The genes are color coded to facilitate comparison with results obtained in the Gata1^{low} animal model: white, altered only in PMF, green altered both in PMF and in untreated Gata1^{low} mice, yellow, altered both in PMF patients and in vehicle-treated Gata1^{low} mice; pink, altered in PMF patients, in untreated and in vehicle-treated Gata1^{low} mice,

* not present in the mouse array.

The mouse microarray data may be retrieved from the GEO website:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44795>

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44797>

The human microarray data may be retrieved from the GEO website:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61818>

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61819>

Table 2

Biological consequences predicted by the David pathway analyses of the TGF- β signaling abnormalities observed in PMF patients

	Nr of genes	Pathway	Genes	Predicted
Marrow	20	Canonical TGF- β signaling	SMAD2, AMHR2, BMP2, BMP4, BMP7, BMPR1A, CHRD, FST, GDF5, GDF6, INHBA, INHBB, ID1, LTBP1, LEFTY1, NODAL, NOG, TGFB3, TGFB1, TGFB2	Osteoblast differentiation $\uparrow$ G1 arrest, apoptosis $\uparrow$ Ubiquitin mediated proteolysis $\downarrow$
	6	MAPK signaling	EVI1, GADD45B, JUN, TGFB3, TGFB1, TGFB2	Apoptosis $\uparrow$ Fibrosis $\uparrow$
	3	Hedgehog signaling	BMP2, BMP4, BMP7	Stress Pathway $\uparrow$
	0	p53 signaling	No hit	-
	5	Cell Cycle	SMAD2, P21CIP1, P27KIP1, GADD45B, TGFB3	G1 arrest, apoptosis $\uparrow$
	6	CML	P21CIP1, P27KIP1, EVI1, TGFB3, TGFB1, TGFB2	G1 arrest, apoptosis $\uparrow$
	12	Pathways in cancer	SMAD2, BMP2, BMP4, P21CIP1, P27KIP1, EVI1, IL6, JUN, STAT1, TGFB3, TGFB1, TGFB2	-
Spleen	18	Canonical TGF- β signaling	SMAD3, SMAD7, SMURF1, ACVRL1, ACVR2A, BMP2, BMPR1B, BMPR2, P21CIP1, FST, ID2, LTBP1, LEFTY1, NODAL, THBS1, TGFB2, TGFB1, TGFB2	G1 arrest, apoptosis $\uparrow$ Fibrosis $\uparrow$ Ubiquitin mediated proteolysis $\downarrow$
	6	MAPK signaling	EVI1, GADD45B, TGFB2, TGFB1, TGFB2, FOS	G1 arrest, apoptosis $\downarrow$ DNA repair and damage prevention $\downarrow$
	0	Hedgehog signaling	No hit	-
	4	p53 signaling	P21CIP1, GADD45B, ID1, THBS1	G1 arrest, apoptosis $\downarrow$ DNA repair and damage prevention $\downarrow$
	5	Cell Cycle	SMAD3, P21CIP1, P15INK4B, GADD45B, TGFB2	G1 arrest, apoptosis $\downarrow$ DNA repair and damage prevention $\downarrow$
	6	CML	SMAD3, P21CIP1, EVI1, TGFB2, TGFB1, TGFB2	G1 arrest, apoptosis $\uparrow$

	Nr of genes	Pathway	Genes	Predicted
	11	Pathways in cancer	SMAD3, BMP2, P21CIP1, P15INK4B, EVI1, IGF1, IL6, TGFB2, TGFB1, TGFB2, FOS	-

Legend: RED over-expressed; GREEN under-expressed