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Shared and Tissue-Specific Expression Signatures between Bone Marrow from Primary Myelofibrosis and Essential Thrombocythemia

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Megakaryocytes have been implicated in the micro-environmental abnormalities associated with fibrosis and hematopoietic failure in the bone marrow (BM) of primary myelofibrosis (PMF) patients, the Philadelphia-negative myeloproliferative neoplasm (MPN) associated with the poorest prognosis. To identify possible therapeutic targets for restoring BM functions in PMF, we compared the expression profiling of PMF BM with that of BM from essential thrombocytopenia (ET), a fibrosis-free MPN also associated with BM megakaryocyte hyperplasia. The signature of PMF BM was also compared with published signatures associated with liver and lung fibrosis. Gene set enrichment analysis (GSEA) identified distinctive differences between the expression profiles of PMF and ET. Notch, K-Ras, IL-8, and apoptosis pathways were altered the most in PMF as compared with controls. By contrast, cholesterol homeostasis, unfolded protein response, and hypoxia were the pathways found altered to the greatest degree in ET compared with control specimens. BM from PMF expressed a noncanonical transforming growth factor β (TGF-β) signature, which included activation of *ID1, JUN, GADD45b,* and genes with binding motifs for the *JUN* transcriptional complex AP1. By contrast, the expression of *ID1* and *GADD45b* was not altered and there was a modest signal for *JUN* activation in ET. The similarities among PMF, liver fibrosis, and lung fibrosis were modest and included activation of integrin-α9 and tropomyosin-α1 between PMF and liver fibrosis, and of ectoderm–neural cortex protein 1 and FRAS1-related extracellular matrix protein 1 between PMF and lung fibrosis, but not TGF-β. These data identify TGF-β as a potential target for micro-environmental therapy in PMF.

Mice overexpressing the transcription factor *JUN*, a gene activated by several inflammatory cytokines including the noncanonical MAPK-dependent transforming growth factor β (TGF-β) [1–3], develop bone marrow (BM), skin, and lung fibrosis and are predisposed to developing fibrosis in response to stresses to the liver and kidney [4]. These observations suggest that in mice, shared mechanisms mediate development of fibrosis across organs. This hypothesis is of great

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clinical relevance as identification of factors that stimulate fibrosis across organs would greatly advance the development of antifibrotic therapies. However, whether shared mechanisms for fibrosis among organs also exist in humans has not yet been established.

Bone marrow fibrosis is the hallmark of the microenvironmental abnormalities responsible for hematopoietic failure in primary myelofibrosis (PMF), the most severe of the Philadelphia-negative myeloproliferative neoplasms (MPNs) [5,6]. It has been hypothesized that PMF fibrosis is induced by TGF-β and possibly other inflammatory cytokines, produced by increased numbers of dysplastic megakaryocytes [7,8]. Fibrosis, however, is not observed in the BM of patients with essential thrombocythemia (ET), a form of MPN that presents with hyperproliferation of polylobulated megakaryocytes rather than the hypolobulated megakaryocytes that characterize PMF.

To identify the fibrosis signature in PMF and to assess whether this signature is similar to that of fibrosis in other organs, we compared the expression profiling of BM from PMF with that of BM from either ET or non-diseased volunteers, as negative controls. The PMF signature was also compared with published signatures of idiopathic pulmonary fibrosis and hepatic fibrosis [9–11]. The data presented indicate the presence of shared, but also distinctive signatures between PMF and ET, and between the fibrotic signature of PMF and those of other organs.

Methods

Human participants

Cryopreserved mononuclear cells from the BM of 15 PMF and three ET patients was provided as de-identified material by IRCCS Policlinico San Matteo, Pavia, Italy. Bone marrow from eight healthy individuals was purchased from AllCells Technology (Oakland, CA). mRNA in amounts sufficient for analyses was obtained from 6 PMF and all ET patients and healthy controls. The clinical data of informative patients at the time of BM harvest are summarized in Table 1. The study was approved by the institutional review board of Policlinico San Matteo, Pavia, Italy (Authorization No. 20110004143, September 26, 2011) and is compliant with the Declaration of Helsinki for Studies Involving Human Subjects. ET7 was analyzed twice, at 3 years postdiagnosis (a) and 1 year later (b).

RNA extraction and microarray analyses

Total RNA was prepared with Trizol (Gibco-BRL, Grand Island, NY) and purified with the Rneasy Mini Kit (Qiagen, Germantown, MD). Hybridization to the microarray Human HT-12_v4 Bead Chip gene expression array (Illumina, San Diego, CA, USA) was performed by the Microarray Resource Facility, Icahn School of Medicine at Mount Sinai. Two sequential microarray analyses were performed. Microarray 1 included 3 PMF patients, two replicate measurements each (PMF1-3, Table 1), and 5 nondiseased controls, one measurement each. Microarray 2 included 3 PMF patients (PMF4 and a pool of PMF5 and PMF6), 3 ET patients (ET7–ET9, Table 1) and 3healthy controls. PMF4 was analyzed in duplicate, the PMF5/PMF6 pool, ET7, ET9, and the healthy controls were measured once. BM from ET7 was analyzed at 3 years (two replicates) and 4 years postdiagnosis. The entire microarray data set is available in the Gene Expression Omnibus database, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124281.

Data analyses

By using the GenomeStudio software (Illumina), the two microarrays were imported into gene cluster text (GCT) files (*GCT file 1* and *GCT file 2*). In total, 47,323 RNA microarray probes were identified with a handful of missing values (of 47,323 probes, 7 were missing from GCT file 1 and *22* from GCT file 2), which was probably due to failure during the import of the microarray data into the GCT files. By use of SAS 9.4 software (SAS, Cary, NC), the two separate files were combined into one GCT file, and the data were normalized with the Illumina Normalizer (Illumina) module in GenePattern (Broad Institute, University of California, San Diego, CA). The data were then analyzed with the sampleFilterPercentP_R2 module of GenePattern and found to be of good quality (correlation to median array >0.95for all samples). The collapsed data by gene (took median) were created with the CollapseDataset module in GenePattern, and identified 31,426 genes. Primary component analysis (PCA) with the PCA module in GenePattern verified that the controls included in GCT files 1 and 2 were similar (Figure 1A). The data reflected the strong separation and clustering of samples,

Table 1. Clinical features of the MPN patients included in the study

ID	Sex	Age at diagnosis	No. of evaluations	Disease duration	IPSS	Fibrosis grade	Mutation (allele burden)	Therapy
PMF1	F	57	2	5 y	Int-2	MF3	JAK2 (86%)	Hydrox
PMF2	F	72	2	2 y	Int-1	MF3	JAK2 (Het)	Hydrox
PMF3	M	81	2	1 y	Int-1	MF1	CALR [del]	No
PMF4	M	38	2	16 y	Int-1	0/1	JAK2 (Het)	Ruxol
PMF5	M	68	1	5 y	Int-1	MF2	CALR [ins]	Ruxol
PMF6	M	54	1	4 y	Int-2	MF1	JAK2 (76%)	Ruxol
ET7a	M	51	2	4 y	High	0	MPL	Hydrox
ET7b	M	51	1	3 y	Low	0	MPL	Hydrox
ET8	M	47	1	4 y	Low	0	JAK2 (Het)	Aspirin
ET9	F	67	1	Diagnosis	High	0	JAK2 (5%)	Aspirin

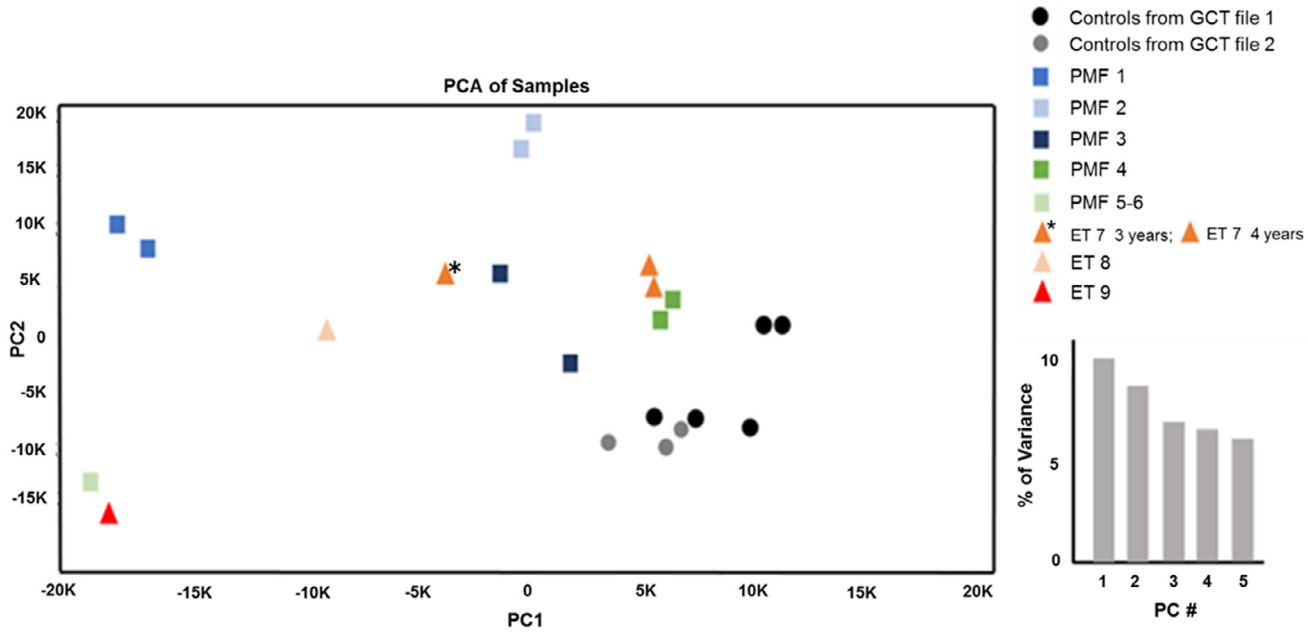

Figure 1. Comparison of the expression profiles of BM from PMF patients, ET patients, and healthy controls. **(A)** Principal component analysis (PCA). To assess the relationship between samples, we performed PCA on the normalized data obtained in duplicate on the same sample in the two data sets. The strong separation and clustering of samples indicate the high reproducibility of data from the same sample. The first PCA reveals differences between controls and patients with MF, about 10% of the variance in the data. **(B)** Comparison of the 100 genes (50 most upregulated and 50 most downregulated) differentially expressed by BM from PMF patients with respect to healthy controls, those from ET patients with respect to healthy controls, and those from PMF patients with respect to ET, as indicated.

demonstrating the quality of the data. The first principal component contains the difference between controls and patients with MF, about 10% of the variance in the data (Figure 1A). Finally, we collapsed the data set by subject (took average) and excluded genes with coefficients of variation (= SD/mean) <0.1, to create the final data set for analysis. Collectively, we analyzed 8,528 genes (after excluding 22,898 noise genes). Gene set enrichment analysis was performed with the GSEA module in GenePattern, utilizing the human gene sets *h.all.v6.1.symbols.gmx*, comparing PMF with control, ET with control, and PMF with ET.

Statistical analyses

The false discovery rate (FDR) were used for multiple comparisons of gene expressions. Genes were considered up- and downregulated when fold changes were >1.4 and <0.7, respectively. In multiple comparison of genes, the level of significance was set at $p < 0.05$ (significant) and $p < 0.1$ (trend).

Results

The expression profiles of the PMF and ET patients were heterogeneous, whereas those of controls were tightly clustered (Figure 1A). As the duplicate measurements of the same patient were also tightly clustered (Figure 1A), the variability among patients likely reflects differences in driver mutations and/or fibrosis levels (International Prognostic Score System int-1–2 and fibrosis grade 1–3) (Table 1). The expression profile of ET7 clustered with that of PMF3 at the 3-year time point and with that of PMF4 at the 4-year time point (Figure 1A). The fact that the top 50 genes differentially expressed in ET7 at the two time points remain the same and do not include any gene differentially expressed between patients and controls (Figure 1B; Supplementary Figure S1, online only, available at www.exphem.org) suggests that differences observed in ET7 at the 3- and 4-year time points underlie variability unrelated to MPN.

Despite the heterogeneity in expression profiles among patients, common abnormalities were observed between patients and healthy controls (Figure 1B and data not shown). In PMF, there were 426–516 transcripts up- and downregulated in the patients, as compared with controls. As expected because of the great frequency of megakaryocytes in PMF, expression of the megakaryocyte-specific transcription factor *GATA1* was significantly upregulated in PMF (1.5-fold higher than in healthy controls, $p = 0.04$). The transcription factor *JUN* (3.0-fold higher than normal controls, $p = 0.01$) and its related genes *JUNB* (1.2-fold, $p = 0.767$) and *FOSB* (2.8-fold, $p = 0.06$) were also among the most enriched genes relative to controls (Figure 1B).

Because the transcription factor complex JUN/FOS binds to consensus sequences defined as AP-1 [12–14], we confirmed that the high levels of *JUN/FOS*

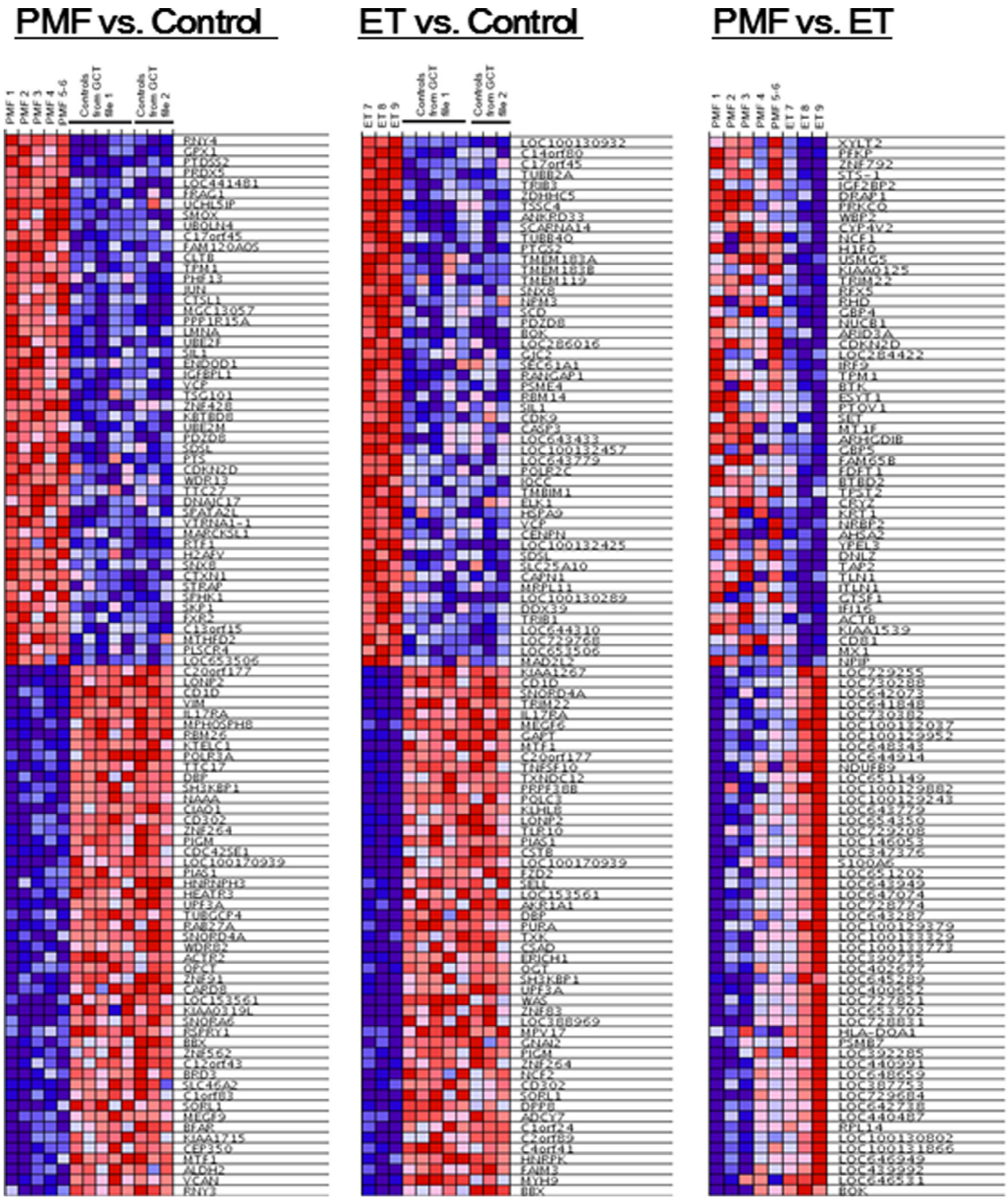

Figure 1. Continued.

expressed by BM from PMF have physiological consequences by determining that the expression of several genes with putative AP-1 binding sites was also significantly altered in the profiling of the BM from the three PMF patients analyzed using microarray 1 (Table 2).

We found that the strong *JUN* signature of PMF BM determined in the current study confirms the high levels of expression of this transcription factor previously described by us by analyzing BM from three additional PMF patients with TGF-β-specific microarrays [15]. It is also consistent with the high *JUN* and *FOS* content detected by immunohistochemistry in stromal cells from BM biopsies of 57 PMF patients [4].

In ET, there were 332–833 transcripts up- and downregulated, with respect to healthy controls (FDR, $p < 0.10$). Of those, 81 and 77 were consistently up- and down-regulated in all patients (Figure 1B). In the case of ET, the gene upregulated the most was *PTGS2* (*prostaglandin-endoperoxide synthase 2,* also known as *COX*), an enzyme involved in the prostaglandin

Table 2. Molecular pathways differentially induced between MF patients and controls (GSEA)

Gene set database	Gene sets induced in MF	NES	*p*	FDR
Genes with AP1-related binding motif in their promoter*	AP1_01	1.71	0.000	0.000
	AP1_Q6	1.68	0.000	0.003
	AP1_Q6_01	1.57	0.000	0.007
	AP1_C	1.56	0.002	0.005
	AP1_Q4	1.54	0.000	0.005
	AP1_Q4_01	1.44	0.000	0.010
	AP1_Q2_01	1.43	0.002	0.009
	AP1FJ_Q2	1.32	0.009	0.025
	AP1_Q2	**1.23**	**0.033**	**0.060**

NES=normalized enrichment score; FDR=false discovery rate. Gene sets with an FDR <0.25 or top 20 are shown. Gene sets with an FDR >0.05 are in boldface.

*In the regions spanning up to 4 kb around transcription start site in the TRANSFAC database, Version 7.4 (http://gene-regulation.com/pub/databases.html).

biosynthetic pathway expressed at high levels in BM and a potent mediator of inflammation [16]. In ET, *JUN* and *FOS* were expressed at levels lower than those in PMF and there was only a trend toward activation (*JUN,* 3.0-fold increase, $p = 0.097$; *FOSB, 4.3*-fold increase, $p = 0.097$) relative to controls.

In our study, mRNA was prepared from BM mononucleated cells thawed after cryopreservation. BM mononuclear cells are a heterogeneous population enriched for hematopoietic (mainly stem/progenitor cells, megakaryocytes, and monocytes) and stromal (mesenchymal stem cells, fibroblasts, and endothelial cells) cells and deprived of erythroid cells and granulocytes by the thawing procedure. Based on histological data, we infer that BM mononuclear cells from PMF and ET contain ~3-fold more megakaryocytes than normal samples, while PMF is slightly enriched for CD34+ cells (2%–3%) than both ET and normal controls (~ 2% in both cases). The overall content of monocytes and stromal cells is instead comparable among samples.

To assess the contribution of stem/progenitor cells to the expression signature of BM mononuclear cells, we compared the expression profile of PMF BM with that of PMF CD34+ cells published by Guglielmelli et al. [17] (Supplementary Table S1, online only, available at www.exphem.org). Three of the 12 genes found differentially expressed in CD34+ cells were excluded as noise before statistical evaluation. Three genes (*CD9, DLK1,* and *NFE-2*) upregulated in Guglielmelli et al. [17] were also found upregulated above threshold (>1.7-fold change) with respect to controls in our study. Therefore, despite their low numbers, we believe that CD34+ cells have contributed to the readouts of our arrays.

It has been suggested that the stromal and hematopoietic cells of BM respond to TGF-β by activating its noncanonical MAPK-dependent and canonical SMAD-dependent signaling pathway, respectively (see Graphical Abstract, online only, available at www.exphem.org). Because *JUN* is an important target of the noncanonical pathway, the strong *JUN* signature detected in PMF suggests that in these patients, TGF-β signaling is mostly active in stromal cells. To confirm that the canonical TGF-β signaling is not active in PMF and to exclude that the observed *JUN* overexpression is mediated by some other inflammatory cytokine [18], the TGF-β gene expression profiling of PMF and ET with respect to controls was compared using GSEA (Figure 2).

The differences observed in the TGF-β data set between samples were in general modest and not consistent. In agreement with the notion that it is not the expression of TGF-β per se but rather its bioavailability in the microenvironment that is increased in PMF [19], TGF-β was not detected in the PMF or ET signature. However, when compared with controls, PMF bone marrow had more TGF-β-related genes (5 genes, 3 upregulated and 2 downregulated) expressed at altered levels than ET (3 genes, all upregulated). In PMF, there was a significant abnormal expression of *HIPK2* (downregulated) and of ID1 and *JUN* (upregulated) and a trend toward greater expression of *FOSB* and *GADD45b.*

In addition, comparison of the TGF-β signature of PMF patients with fibrosis grade 3 (PMF1 and PMF2) with that of patients with fibrosis grade 0–2 (PMF3–PMF6) (Supplementary Table S2, online only, available at www.exphem.org) indicates that the upregulation of the expression of the non-canonical TGF-β target genes *FOSb, GADD45b* and *ID1/2* increases with disease progression. Interestingly, BM from PMF grade 3 patients also expresses greater levels of *HIPK2, homeodomain-interacting protein kinase 2*, a gene encoding a serine/threonine protein kinase that interacts with homeodomain transcription factors [20,21] and is activated, by overexpression or loss of heterozygosity, in animal models of kidney fibrosis [22–24] and in patients with idiopathic pulmonary fibrosis [25]. By contrast, in ET there was a modest TGF-β signature that included a trend toward *FOSB, JUN,* and *IL6* upregulation with respect to controls.

Activation of the noncanonical p38/ERK-dependent TGF-β signature *JUN, ID1,* and *GADD45* [26,27] was already reported both in PMF patients and in the *Gata1^{low}* animal model of the disease [28]. In addition, expression of these genes was normalized in the mouse model by treatment with a TGF-β receptor 1 kinase inhibitor [28], which also rescued their myelofibrosis phenotype. We believe that overexpression of these

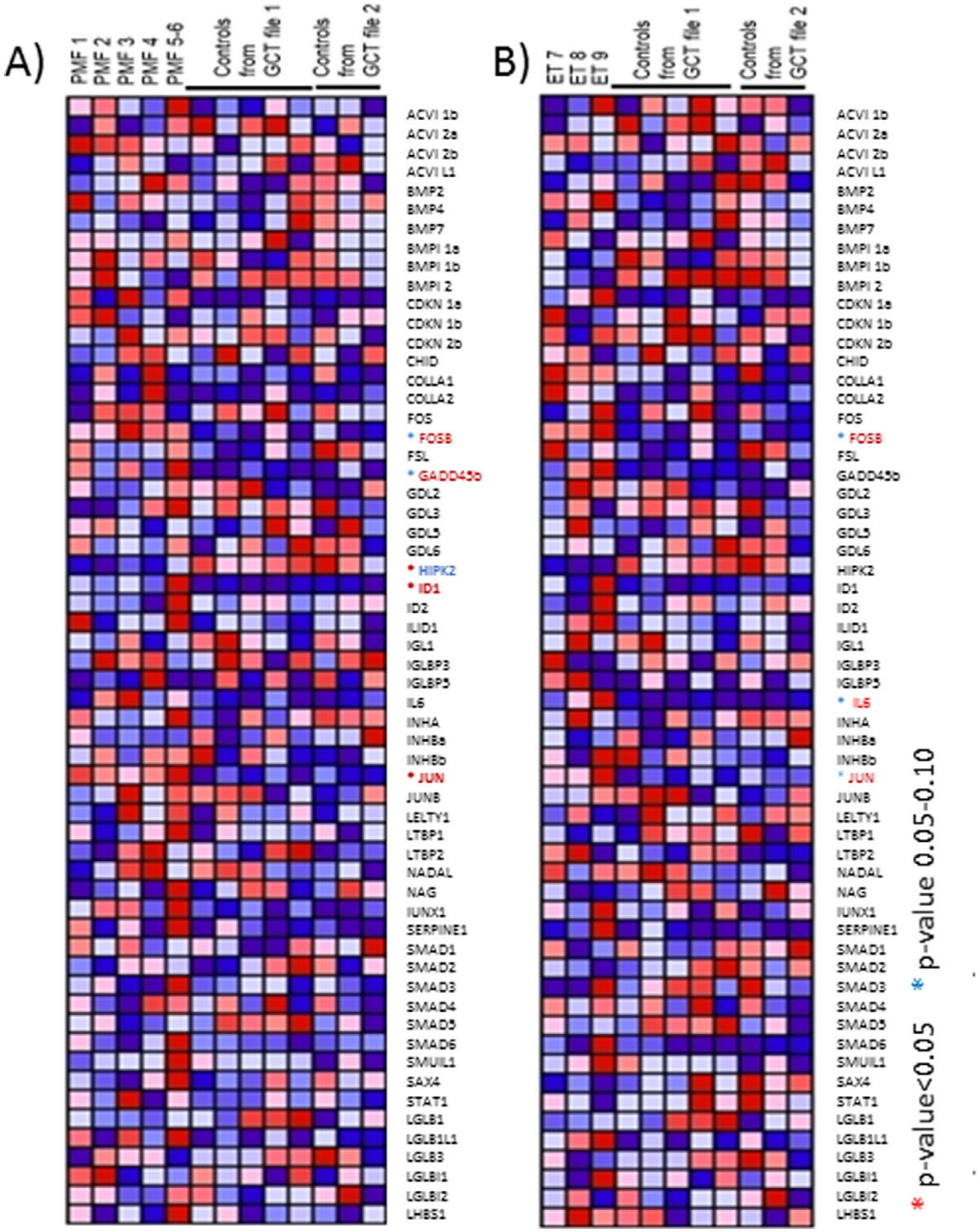

Figure 2. Comparison of the expression profiles of BM from **(A)** PMF and **(B)** ET patients versus healthy controls indicates the presence of an activated noncanonical TGF-β signature only in PMF. *Red* and *blue* indicate genes the expression of which is significantly or has a trend to be overexpressed and underexpressed, respectively, with respect to controls.

genes observed in PMF BM is an indication that the stromal cells are being activated by TGF-β to produce fibrosis (see Graphical Abstract, online only, available at www.exphem.org).

By contrast, the target genes expected to be activated by the canonical TGF-β signaling were either expressed at normal levels or downregulated in BM of PMF patients. These genes included *CDKN1b* and

HIPK2. *CDKN1b*, cyclin-dependent kinase inhibitor 1b, encodes p27Kip1, which induces normal hematopoietic stem/progenitor cells into quiescence in response to TGF-β [29,30] and was also found to be downregulated in BM from PMF patients by Ciaffoni et al. [15] (see Graphical Abstract, online only, available at www.exphem.org). We believe that downregulation of *CDKN1b* in BM of PMF reflects the paucity or insensitivity [31] of hematopoietic cells that respond to TGF-β in PMF BM.

Support for the hypothesis that the expression signatures we have identified reflect the disease status of the patient comes from the observation that *IL-8* was upregulated in the BM signature of PMF but not in that of ET. The overexpression of *IL-8* in PMF BM is in agreement with recent observations indicating that the plasma of PMF patients, but not that from ET, contains greater levels of IL-8 than normal, which correlates with disease prognosis [32].

The differences in expression profiling between PMF and ET were further characterized by pathway analyses with GSEA (Table 3). The expression pathways altered in PMF and ET were quite distinct. *Notch* signaling was the most enriched pathway in PMF (NES = 1.62, $p = 0.046$), followed by *K-RAS* (NES = 1.51, $p = 0.036$) and apoptosis (NES = 1.47, $p = 0.121$) as compared with healthy controls. In contrast, cholesterol homeostasis was the most enriched pathway in ET (NES = 1.62, $p = 0.00$), followed by unfolded protein response (NES = 1.18, $p = 0.203$). *Notch* and *K-RAS* were enriched in PMF even when the data were compared with those for ET. The individual genes abnormally expressed in each pathway are summarized in Table 4.

To confirm the hypothesis that the high levels of *GATA1* expression detected in PMF are due to an increased frequency of immature megakaryocytes, the expression signature of PMF was compared with that of normal controls and ET using the list of 26 megakaryocyte-specific genes described by Chen et al. [33] and Psaila et al. [34] (Supplementary Table S3, online only, available at www.exphem.org). Twelve genes were overexpressed, and 3 genes were underexpressed in PMF with respect to normal. In addition to *GATA1*, examples of genes overexpressed in PMF are *MPL*, the receptor for thrombopoietin; *CD36,* platelet glycoprotein IV or thrombospondin receptor; and *GP9*, glycoprotein IX, which are expressed at higher levels in immature megakaryocytes [35]. In contrast, genes expressed at greater levels by mature megakaryocytes, such

Table 3. GSEA enrichment terms from Gene Ontology using GSEA for PMF versus control, ET versus control, and PMF versus ET

Name of enrichment term	Size	ES	NES	NOM p-val	FDR p-val	FWER p-val
				PMF versus control		
NOTCH_SIGNALING	17	0.57	1.62	0.046	0.795	0.292
KRAS_SIGNALING_DN	38	0.48	1.51	0.036	0.846	0.465
APOPTOSIS	116	0.43	1.47	0.121	0.742	0.545
ESTROGEN_RESPONSE_EARLY	80	0.33	1.46	0.041	0.610	0.561
HEME_METABOLISM	158	0.58	1.43	0.192	0.558	0.596
P53_PATHWAY	120	0.36	1.40	0.079	0.536	0.631
TNFA_SIGNALING_VIA_NFKB	151	0.53	1.33	0.236	0.647	0.745
CHOLESTEROL_HOMEOSTASIS	49	0.41	1.32	0.190	0.605	0.757
HYPOXIA	116	0.33	1.31	0.102	0.559	0.771
				ET versus control		
CHOLESTEROL_HOMEOSTASIS	49	0.48	1.62	0.000	0.461	0.204
UNFOLDED_PROTEIN_RESPONSE	85	0.30	1.18	0.203	1.000	0.887
HYPOXIA	116	0.28	1.16	0.210	1.000	0.915
ANGIOGENESIS	17	0.44	1.16	0.272	1.000	0.915
MYOGENESIS	64	0.28	1.07	0.352	1.000	0.952
ANDROGEN_RESPONSE	59	0.30	1.06	0.390	1.000	0.964
MTORC1_SIGNALING	149	0.26	1.06	0.343	1.000	0.964
COAGULATION	59	0.30	1.03	0.363	1.000	0.964
EPITHELIAL_MESENCHYMAL_TRANSITION	81	0.32	1.02	0.599	1.000	0.967
				PMF versus ET		
KRAS_SIGNALING_DN	38	0.53	1.67	0.000	0.272	0.113
WNT_BETA_CATENIN_SIGNALING	19	0.57	1.64	0.035	0.187	0.196
PEROXISOME	54	0.41	1.61	0.000	0.198	0.257
INTERFERON_ALPHA_RESPONSE	81	0.69	1.44	0.034	0.627	0.615
APICAL_JUNCTION	73	0.46	1.35	0.000	0.939	0.781
NOTCH_SIGNALING	17	0.42	1.32	0.107	0.985	0.886
INTERFERON_GAMMA_RESPONSE	152	0.53	1.26	0.218	1.000	0.886
REACTIVE_OXIGEN_SPECIES_PATHWAY	36	0.35	1.17	0.197	1.000	0.916
BILE_ACID_METABOLISM	44	0.30	1.14	0.320	1.000	0.931

ES=enrichment score; NES=normalized enrichment score

Table 4. GSEA enrichment terms from Gene Ontology using GSEA for PMF versus control, ET versus control, and PMF versus ET*

Pathways (number of genes)	Genes abnormally expressed
	Differences between PMF and control
NOTCH_SIGNALING (17)	Overexpressed: ***SKP1,*** *HES1, FZD5*
KRAS_SIGNALING_DN (38)	Overexpressed: ***BTG2, ADRA2C,*** *FGFR3, IFNG* Underexpressed: **MTHFR,** BARD1
APOPTOSIS (116)	Overexpressed: ***GPX1,*** *JUN,* ***LMNA, CD69, ETF1,*** *BTG3,* ***PMAIP1, PDGFRB,*** *EGR3,* ***BTG2, IL1B, SMAD7,*** *TNF,* ***DAP,*** *SAT1, GADD45B, SQSTM1* Underexpressed: ***CD44, DPYD, RNASEL,*** *CASP2,* ***CFLAR,*** *DFFA*
ESTROGEN_RESPONSE_EARLY (80)	Overexpressed: ***ENDOD1,*** *FHL2,* ***PMAIP1,*** *EGR3, NXT1, HES1, MUC1,* ***SLC7A5,*** *MAST4, FASN,* ***SLC37A1,*** *PPIF* Underexpressed: ***CD44, ISG20L2, RAB31, ADD3,*** *XBP1,*
HEME_METABOLISM (158)	Overexpressed: ***SMOX, ENDOD1, GATA1,*** *ELL2,* ***TAL1,*** *CIR1, SLC10A3, OPTN,* ***SLC11A2, PQLC1, ACP5, BTG2, ARHGEF12,*** *MPP1, PIGQ, OSBP2* Underexpressed: *CTSB, ALDH6A1*
	Differences between ET and control
CHOLESTEROL_HOMEOSTASIS (49)	Overexpressed: *TRIB3, SCD, DHCR7, LGMN* Underexpressed: ***FAM129A,*** *FBXO6,* ***ATXN2***
UNFOLDED_PROTEIN_RESPONSE (85)	Overexpressed: *TUBB2A, HSPA9, SLC7A5, ZBTB17, ATP6V0D1* Underexpressed: ***SDAD1, SPCS1,*** *TTC37,* ***ATF6, SEC31A, IMP3,*** *PARN*
HYPOXIA (116)	Overexpressed: *PRDX5, JMJD6, PLIN2, ZFP36, PPP1R15A, GRHPR, JUN, DDIT3, GAPDH, SDC4* Underexpressed: ***MYH9, MAP3K1, CCNG2, BCL2, PLAC8, FOXO3, IRS2, PGM1, PRKCA, GLRX,*** *PFKP, NAGK, ENO1*
ANGIOGENESIS (17)	Overexpressed: ***VCAN***
MYOGENESIS (64)	Overexpressed: *SCD, SPHK1* Underexpressed: ***MYH9, OCEL1, VIPR1, MEF2C,*** *PLXNB2*
	Differences between PMF and ET
KRAS_SIGNALING_DN (38)	No hit
WNT_BETA_CATENIN_SIGNALING (19)	No hit
PEROXISOME (54)	No hit
INTERFERON_ALPHA_RESPONSE (81)	No hit
APICAL_JUNCTION (73)	Underexpressed: ***ACTB,*** *TGF-β1*

*Genes with statistically significant fold changes are in boldface.

as *PF4*, were expressed at levels similar to those in controls (1.14-fold change, $p = 0.37$). The differences in gene expression (4 overexpressed and 3 underexpressed) between ET and normal controls were modest and largely overlapped with those observed in PMF. The only uniquely abnormal gene in ET was *DHRS3*, which encodes short-chain dehydrogenase/reductase 3. The reduced number of abnormalities observed in ET confirms that in this disease, the maturation of megakaryocytes is overall normal. Of interest, *CD47*, the "don't eat me" signal [36], was slightly downregulated above threshold with respect to normal both in ET (0.72-fold change, $p = 0.0025$; Supplementary Table S3, online only, available at www.exphem.org) and PMF (0.82-fold change, $p = 0.068$), a further indication that the immune control is impaired in myeloproliferative neoplasms [37]. Lastly, comparison between the megakaryocyte gene signatures of PMF and ET largely identifies the same gene expression differences (11 upregulated) observed between PMF and controls (Supplementary Table S3, online only, available at www.exphem.org).

The expression profiles of the BM from PMF patients were also compared with the gene expression signatures from fibrotic tissues or cells, including activated hepatic stellate cells (HSCs, 100 upregulated and 100 downregulated [8], 100 upregulated [9]) and tissues from patients with idiopathic pulmonary fibrosis (100 upregulated and 100 downregulated, GSE47460 [10]). The comparison with these published signatures of liver fibrosis and lung fibrosis indicated modest similarity. Some common genes included activated integrin-α9 (*ITGA9*) and tropomyosin-α1 (*9TPM1*), when compared with liver fibrosis signatures, and ectoderm −neural cortex protein 1 (*ENC1*) and FRAS1-related extracellular matrix protein 1 (*FREM1*) when compared with the lung fibrosis signature (Figure 3).

Discussion

One limitation of our study is the limited number of patients included in the analyses. In this regard, it is common experience that, as a result of the underlying fibrosis, the cell content of most BM biopsies from PMF patients is low and does not provide mRNA in amounts sufficient for analyses. From a total of 15 PMF BM samples processed, we obtained mRNA for microarray analyses from only 6 patients (Table 1). This limitation prevented us from validating the expression patterns identified by quantitative real-time reverse transcription polymerase chain analyses. However, because of their rarity, the availability of these

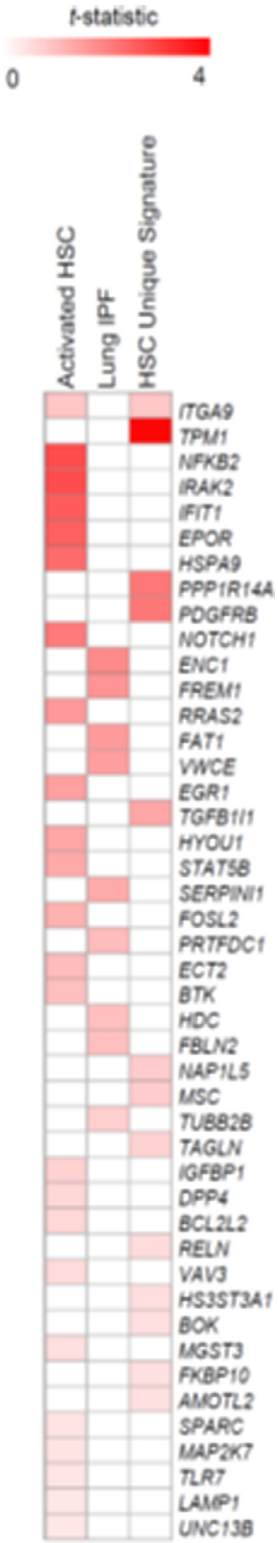

Figure 3. Comparison of the expression signatures of the BM from PMF patients (this study) with published signatures of hepatic stellate cells (activated HSCs [10] and HSCs with unique signatures [8,9]) that are responsible for liver fibrosis and idiopathic pulmonary fibrosis [10]. The color indicates the statistical strength of the similarity between the abnormalities.

data in a public database will facilitate studies on marrow fibrosis using more advanced techniques such as single-cell profiling.

In summary, this study confirms the presence of a noncanonical TGF-β signature in BM from PMF, supporting the hypothesis that inhibitors of TGF-β represent attractive candidates for therapies targeting the micro-environment in PMF.

Acknowledgments

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Conflict of interest disclosure

The authors have no conflicts of interest to declare.

Authorship contributions

GI analyzed the data and wrote the article. NF, HH, YH, and MM analyzed the data. LV prepared mRNA samples and coordinated the microarray analyses with the ISMMS core facility. GB and VR provided the patient samples. MP, SLF, and RH wrote the article. ARM designed the study, interpreted data, and wrote the article. All authors read and approved the submitted article.

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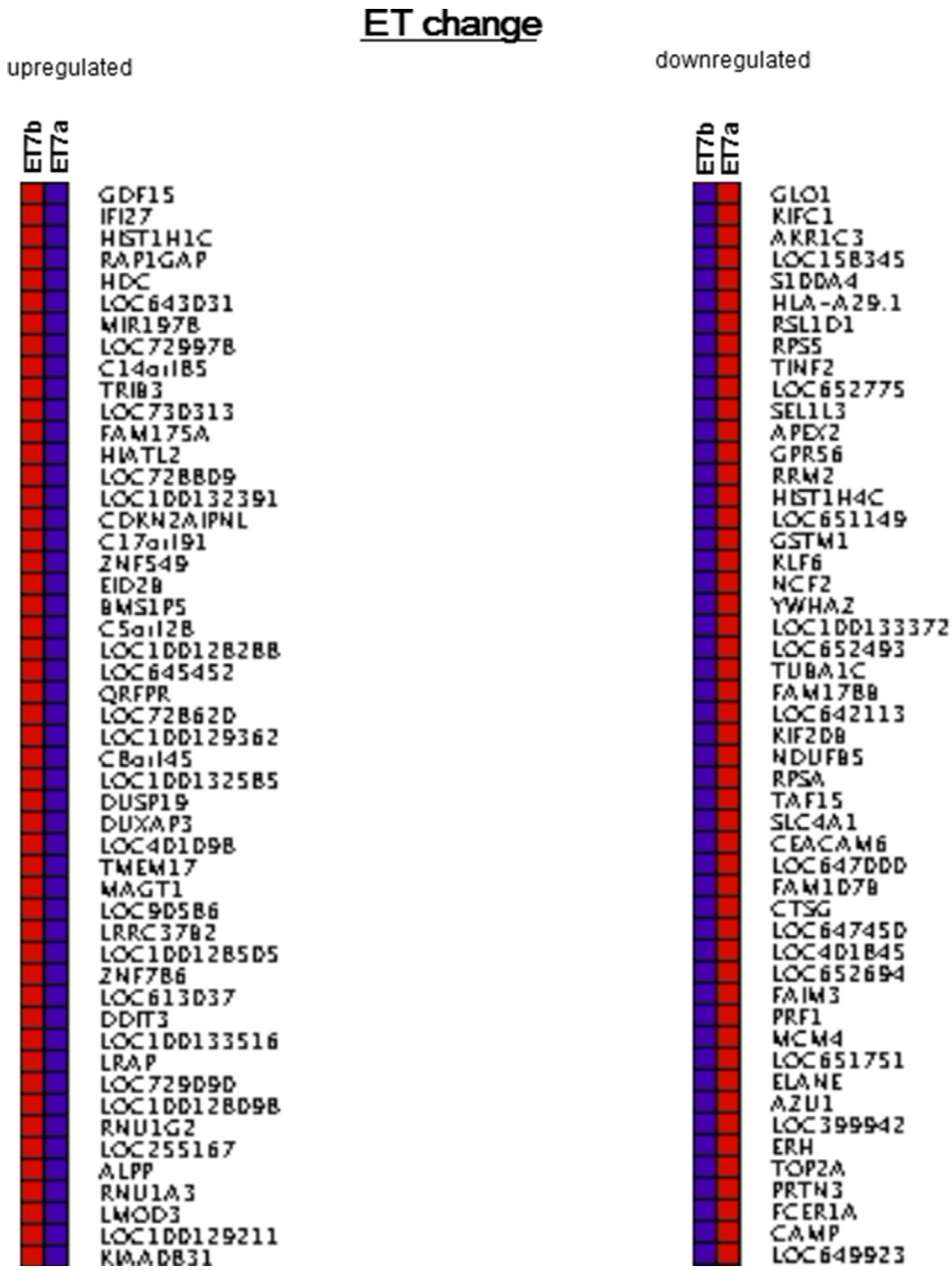

Figure S1. Comparison of the genes (50 most upregulated and 50 most downregulated) differentially expressed by the BM of ET7 at the 3 and 4 year post-diagnosis time point. Canonical SMAD-dependent TGF-β signaling inhibits adult hematopoiesis by inducing HSC into quiescence[38], by eliciting a Smad5-dependent inhibition of progenitor cell proliferation[39] by increasing the length of G1 by reducing G1 cyclin and cyclin-dependent protein kinases[40,41] and triggering Smad4-signaling which accelerates terminal erythroid maturation[42].

Table S1. (new): Comparison of the expression signature of the bone marrow from PMF patients with the CD34$^+$ cell signature published by Guglielmelli et. all[17]. Values reported to be up and down-regulated in [17] are in red and blue fonts, respectively. Fold changes down by >0.7 and up >1.4 in our study are in bold blue and red fonts, respectively.

	Fold change PMF vs. Control	p-value (FDR)
CD9	**1.655**	0.138
CD164	1.009	0.963
GAS2	1.003	NA[#]
DLK1	**2.568**	0.692
CDH1	1.338	0.422
NFE2	**1.749**	0.107
CXCR4	1.115	0.776
WT1	1.045	NA[#]
HMGA2	1.001	NA[#]

*GAS2, WT1, and HMGA2 were excluded as noise prior to statistical analysis

Table S2. (New): Comparison of the TGF-β expression signature between patients with PMF stage 3 (two patients, PMF1 and 2) versus stage 0- 2 (four patients, PMF 3, 4, 5 and 6). Genes with fold changes down by >0.7 and up >1.4 are highlighted

Genes related to TGF-beta pathway	Fold change (Stage 3 vs. Stage 0,1,2)	p-value FDR(BH)
ACVR 2B	0.990101851	**0.001966**
BMPR 2	0.902049375	**0.001966**
CDKN 1B	**1.48655251**	**0.001966**
FOSB	**1.778412847**	0.620556
GADD45B	**1.598874421**	0.947975
GDF5	0.988537388	**0.001966**
ID1	**1.506047446**	0.947975
ID2	**1.462667752**	0.947975
JUNB	**1.705900713**	0.620556
SMAD5	0.979377463	**0.001966**
STAT1	**1.461267978**	0.947975
THBS1	**2.284726857**	0.947975

Table S3. (NEW): Megakaryocytes specific genes differentially expressed in bone marrow from PMF vs controls, ET vs control and PMF vs ET.

Genes	Fold Change (PMF vs. control)	p-value FDR(BH)
GATA1	**1.521918776**	**0.023395321**
GP1BA	**1.787800252**	0.070185963
ITGA2B	**2.599427333**	0.093581284
ITGB3	**1.72972681**	0.093581284
GP9	**3.855189333**	0.101751078
TMOD1	**1.897211628**	0.11665167
CD9	**1.655041183**	0.070185963
NFIB	**1.570451691**	0.11229754
MPL	**1.931897638**	0.101751078
VWF	**2.210376106**	0.101751078
ANK1	**1.531336841**	0.123908552
THBS1	**1.852515622**	0.459489055
LEF1	**0.663498003**	0.399280144
CD36	**0.640969323**	0.068626275
CD44	**0.557611515**	**0.00519896**
Genes	**Fold Change (ET vs. control)**	**p-value FDR(BH)**
DHRS3	**1.720285887**	0.221822302
ITGA2B	**2.118250227**	0.340160539
GP9	**2.74502003**	0.386322735
ITGB3	**1.798746775**	0.475568044
CD44	**0.69034498**	0.340160539
CD47	**0.726603431**	**0.00259948**
LEF1	**0.386757833**	**0.00259948**
Genes	**Fold Change (PMF vs. ET)**	**p-value FDR(BH)**
GP1BA	**1.556416177**	0.91857418
NFIB	**1.465176765**	0.91857418
MPL	**1.853773816**	0.91857418
CD9	**1.458962813**	0.91857418
VWF	**1.793364058**	0.91857418
TMOD1	**1.713192123**	0.91857418
LEF1	**1.71553863**	0.91857418
THBS1	**1.417220461**	0.960607878
GP9	**1.404430311**	0.91857418

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The expression signature of PMF BM suggests that in this organ TGF-β activates a non-canonical MAPK dependent signaling that induces stromal cells to produce fibrosis. The diagram illustrates target genes reported to be activated (red arrows) or repressed (blue arrows) by the non-canonical and canonical TGF-β signaling, respectively, in stromal and hematopoietic cells of the bone marrow, and of their respective predicted biological effects. In red and blue fonts, instead, are summarized the activated and repressed state of these same target genes in the arrays of BM from PMF

patients described here. Black fonts, instead, indicate genes found to be expressed at normal levels in PMF. There is a good correlation between predicted and detected expression state of the genes of the non-canonical TGF-β signaling (4 out of 5 target genes), suggesting that in PMF bone marrow stromal cells are activated by TGF-β. By contrast there is no agreement between the predicted and the detected expression state of targets of the canonical pathway (0 out of 8 targets), supporting the hypothesis that the malignant hematopoietic cells do not respond to TGF-β.