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Aberrant let7a/HMGA2 signaling activity with unique clinical phenotype in *JAK2*-mutated myeloproliferative neoplasms

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ABSTRACT

High mobility group AT-hook 2 (HMGA2) is an architectural transcription factor that is negatively regulated by *let-7* microRNA through binding to it's 3'-untranslated region. Transgenic mice expressing *Hmga2* with a truncation of its 3'-untranslated region has been shown to exhibit a myeloproliferative phenotype. To decipher the *let-7-HMGA2* axis in myeloproliferative neoplasms, we employed an *in vitro* model supplemented with clinical correlation. Ba/F3 cells with inducible *JAK2*V617F expression (Ton.JAK2.V617F cells) showed upregulation of HMGA2 with concurrent *let-7a* repression. Ton.JAK2.V617F cells treated with a *let-7a* inhibitor exhibited further escalation of *Hmga2* expression, while a *let-7a* mimic diminished the *Hmga2* transcript level. *Hmga2* overexpression conferred *JAK2*-mutated cells with a survival advantage through inhibited apoptosis. A pan-JAK inhibitor, INC424, increased the expression of *let-7a*, downregulated the level of *Hmga2*, and led to increased apoptosis in Ton.JAK2.V617F cells in a dose-dependent manner. In samples from 151 patients with myeloproliferative neoplasms, there was a modest inverse correlation between the expression levels of *let-7a* and *HMGA2*. Overexpression of *HMGA2* was detected in 29 (19.2%) of the cases, and it was more commonly seen in patients with essential thrombocythemia than in those with polycythemia vera (26.9% *vs.* 12.7%, *P*=0.044). Patients with upregulated *HMGA2* showed an increased propensity for developing major thrombotic events, and they were more likely to harbor one of the 3 driver myeloproliferative neoplasm mutations in *JAK2*, *MPL* and *CALR*. Our findings suggest that, in a subset of myeloproliferative neoplasm patients, the *let-7-HMGA2* axis plays a prominent role in the pathogenesis of the disease that leads to unique clinical phenotypes.

Introduction

Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs), which include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are a group of clonal disorders of the hematopoietic system characterized by excessive production of differentiated myeloid cells. With the discoveries of underlying driver mutations in *JAK2*, *MPL*, and calreticulin (*CALR*) that together account for 90% of patients with myeloproliferative neoplasm (MPN), it is now clear these conditions are characterized by dysregulated JAK-

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STAT (signal transducer and activator of transcription) signaling pathways.[1] However, none of these mutations have been proven to be specific to disease subtype. As a result, they cannot be used in the molecular classification of MPNs. In addition, it remains unclear why the same acquired mutation in one of these genes causes similar clinical entities with distinct phenotypes.

Some studies allude to the fact that *JAK2*V617F confers only a weak growth advantage to hematopoietic stem cells.[2,3] An emerging hypothesis suggests that several cooperating genetic hits might be required to induce disease and allow progression, as mutations in signaling molecules are not sufficient for disease development in humans.[4] There are quite a few other genetic alterations identified in MPN;[5] among them, several are implicated in epigenetic regulation, either in histone modifications or in DNA methylation control.[6] Studies have shown that these epigenetic regulator genes and *JAK2* mutations are synergistic by combining an early and late amplification, with mutation of the former mainly expanding the hematopoietic progenitor cells, whereas *JAK2*V617F mainly expands the mature fraction.[4] Therefore, it is widely postulated that epigenetic regulation might also play an important role in the pathogenesis of MPN.

The high mobility group A2 (*HMGA2*) gene codes for a non-histone protein that has no intrinsic transcriptional activity, but can modulate transcription by altering the chromatin architecture.[7,8] The HMGA2 protein, highly expressed during embryogenesis but diminished in normal adult tissues, is thought to play an essential role in self-renewal and the control of differentiation of embryonic stem cells.[9] However, high levels of HMGA2 are found in various tumors, especially those of mesenchymal origin.[10] The 3'-untranslated region (3'-UTR) of *HMGA2* contains 7 sequences complementary to the *let-7* microRNA (miRNA), which negatively regulates HMGA2 expression.[11] In some tumors, rearrangement around the region of chromosome 12q14-15, the location of the *HMGA2* gene, can lead to a deletion of the *HMGA2* 3'-UTR and loss of *let-7* binding sites. This results in overexpression of a full-length or truncated HMGA2 protein which promotes tumor formation.[2]

Guglielmelli *et al.* first reported the association between *HMGA2* upregulation and MPNs. In their seminal work studying the molecular profiling of $CD34^+$ cells in PMF, they found that abnormal expression of HMGA2 was dependent on the presence of *JAK2*V617F mutation.[12] Subsequently, Ikeda and colleagues demonstrated that transgenic mice expressing 3'UTR-truncated *Hmga2* (Δ*Hmga2*) cDNA exhibited a myeloproliferative phenotype.[13] Enforced expression of Δ*Hmga2* led to a proliferative advantage in hematopoietic stem and progenitor cells. However, in spite of these studies, there are only scarce data available on the frequencies of dysregulated *HMGA2* signaling activity in MPN patients, which severely limits the kinds of conclusions one can draw. Moreover, it remains unclear how *HMGA2* and *JAK2*V617F interact with each other, and whether upregulation of *HMGA2* plays specific roles in the pathogenesis of *JAK2*-mutated MPNs.

In the study herein, we aimed to address some of these issues. An *in vitro* model was employed to elucidate the correlation between *JAK2*V617F and *HMGA2* expression. Furthermore, the phenotypic influences of *HMGA2* overexpression on *JAK2*-mutated MPNs and the cause of *HMGA2* upregulation were also explored.

Methods

Study population and mutational analysis

Relevant information on the patient enrollment, diagnosis,[14] treatment,[15] definition of events,[16,17] and measurement of survival are listed in the *Online Supplementary File*. All participants provided informed consent in accordance with the Declaration of Helsinki. The study was approved by our Institutional Review Board. The detection of *JAK2*V617F, *JAK2* Exon 12, *CALR*, and *MPL* mutations in clinical samples was performed as previously described.[18]

Cell lines and doxycycline induction

Interleukin-3 (IL-3)-dependent Ba/F3 cells with inducible expression of *JAK2*V617F (Ton.JAK2.V617F) or wild-type (WT) *JAK2* (Ton.JAK2.WT) were kindly provided by Professor Gregor Hoermann and Professor Matthias Mayerhofer (Medical University of Vienna, Austria). The expression of *JAK2* was induced by the addition of doxycycline (1μg/ml). The cells were maintained in IL-3 throughout the experiments until 3 hours

Table 1. Clinical and laboratory features of 151 patients with myeloproliferative neoplasm, stratified by expressional status of the *HMGA2* gene.

	HMGA2(-) N=122	HMGA2(+) N=29	*P*
Age, years	59.5 ± 16.1	65.7 ± 14.4	0.058
Female, no. (%)	50 (41.0%)	19 (65.5%)	**0.017**
WBC count, x 10^9/L	13.6 ± 9.1	13.9 ± 6.2	0.873
Hemoglobin, g/L	15.3 ± 4.1	14.5 ± 3.7	0.345
PLT count, x 10^9/L	576 ± 414	789 ± 445	**0.016**
LDH, U/L	287 ± 145	322 ± 264	0.368
Marrow Cellularity, %	72.9 ± 20.5	68.5 ± 21.0	0.449
Splenomegaly, no. (%)	49 (45.8%)	17 (58.6%)	0.220
Major thrombosis, no. (%)	19 (15.6%)	10 (34.5%)	**0.021**
Diagnosis			
PV (n=63)	55 (87.3%)	8 (12.7%)	(PV *vs.* ET) **0.044**
ET (n=67)	49 (73.1%)	18 (26.9%)	(ET *vs.* PMF) 0.542
PMF (n=17)	14 (82.4%)	3 (17.6%)	(PV *vs.* PMF) 0.693
MPN, unclassifiable (n=4)	4 (100%)	0	
Disease risk^ in PV/ET patients			**0.026**
Low-risk	45	5	
High-risk	59	21	
MF Transformation*, no. (%)	5 (4.8%)	1 (3.8%)	0.875
Driver mutation#			**0.051**
Present	94 (77.0%)	27 (93.1%)	
Absent	28	2	
JAK2 mutation *versus* triple negative			**0.040**
JAK2 mutated, no. (%)	78 (73.6%)	22 (91.7%)	
Triple negative	28	2	

^Disease risk stratified according to ELN (European LeukemiaNet) guideline.[15] *MF transformation: Transformation to post-PV or post-ET myelofibrosis in PV & ET patients. # Driver mutations included *JAK2*V617F, *JAK2* Exon12, *CALR*, and *MPL* mutation. Information regarding thrombosis included events at diagnosis and during follow up. MF transformation was assessed during follow up. Other hematological and clinical parameters were collected at diagnosis. Values are reported as mean ± SD unless otherwise indicated. *P* values with statistically significant differences are shown in bold. WBC: white blood cell; PLT: platelet; LDH: lactate dehydrogenase; PV: polycythemia vera; ET: essential thrombocythemia; PMF: primary myelofibrosis; MPN: myeloproliferative neoplasms; MF: myelofibrosis.

before they were subjected to real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot analysis. Sources of other cells used are listed in the *Online Supplementary File.*

Gene silencing by small interfering RNA transfection, miRNA ectopic expression and inhibition

The small interfering RNA (siRNA) oligos that directed against the mouse *Hmga2* messenger RNA (mRNA, si*Hmga2*) or a scrambled sequence (*siScr*) were purchased from Sigma-Aldrich. Small oligos that either mimic or inhibit endogenous *let-7a* were purchased from ABI (mirVana, Thermo Fisher Scientific Inc.). All the transfection was performed using X-tremeGENE siRNA Transfection Reagent (Roche) according to the manufacturer's specifications. The efficiency of various siRNA oligos is demonstrated in the *Online Supplementary Figure S1*. The *si-1 Hmga2* siRNA (*siHmga2*) was considered an ideal option and chosen for further experiments. The oligo concentrations used for *let-7a* inhibition were 0.2 and 0.5 nM according to the manufacturer's suggestion, whereas 0.5 nM was used for the ectopic expression of *let-7a*. All the cells were harvested and validated by qRT-PCR or western blotting after 48 hours of transfection.

Cell viability and apoptosis assays

Cell survival was measured by the XTT assay according to the manufacturer's instructions (Biological Industries). The percentages of apoptotic cells were determined using a FACS Canto II flow cytometer (Becton, Dickinson and Company) after the cells were treated with a 7-AAD and PE-Annexin V Apoptosis Detection Kit (BD Pharmingen). Each data result and its respective error bar were measured by 3 independent experiments run in triplicate.

Growth inhibition assay

The pan-JAK inhibitor INC424 (ruxolitinib) was kindly provided by Novartis Pharmaceuticals. INC424 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM as the stock solution. 0.5% DMSO was added to the culture medium as control. Candidate cells were exposed to various concentrations of hydroxyurea (Bristol-Myers Squibb) or INC424 for 72 h. The drug

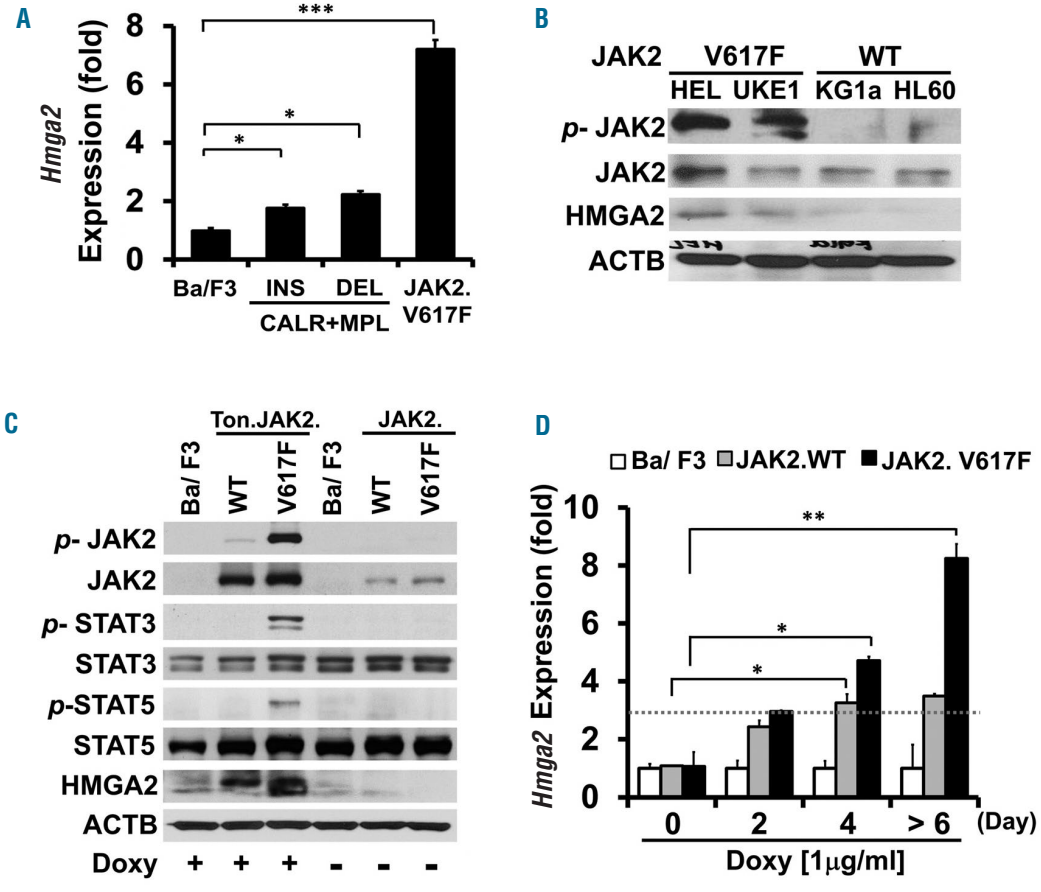

Figure 1. The levels of HMGA2 expression in cells with various JAK-STAT signaling activity. (A) Quantitative RT-PCR analysis of *Hmga2* transcript levels in parental Ba/F3 cells, stable Ba/F3 cells co-transfected with *MPL* and either type 1 (deletion; DEL) or type 2 (insertion; INS) *CALR* mutant, and stable, inducible Ton.JAK2.V617F cells. The Ton.JAK2.V617F cells were treated with doxycycline (1 μg/ml) for at least 6 days before being subjected to analysis. Representative data from three independent experiments are presented. The error bars show the standard deviation (± SD) of three independent experiments. Asterisk indicates statistical significance (*t*-test; **P*<0.01; ****P*<0.0001). (B) Western blot analysis of JAK2, phosphorylated JAK2 (p-JAK2), and HMGA2 levels in 4 human acute myeloid leukemia cell lines: *JAK2*V61F-carrying HEL and UKE-1 cells, and *JAK2*-unmutated KG-1a and HL-60 cells. (C) The expression levels of JAK2, p-JAK2, STAT3, p-STAT3, STAT5, p-STAT5, and HMGA2 proteins in stable, inducible Ton.JAK2.WT and Ton.JAK2.V617F cells. Cells were treated with doxycycline (1 μg/ml; for at least 6 days) before being harvested concurrently with untreated controls for subsequent analysis. (D) Quantitative RT-PCR analysis of *Hmga2* transcripts in parental Ba/F3, Ton.JAK2.WT and Ton.JAK2.V617F cells at baseline as well as 2, 4, and 6 days after addition of doxycycline (1 μg/ml), respectively. The error bars show the standard deviation of three independent experiments. Asterisk indicates statistical significance (*t*-test; **P*<0.01; ***P*<0.001). WT: wild-type.

concentrations that inhibited cell growth by 50% (IC_{50}) and 90% (IC_{90}) were determined by the XTT assay.

Others

Protocols on western blotting, qRT-PCR, and fluorescence *in situ* hybridization (FISH) are all listed in the *Online Supplementary File*.[19]

Statistical analysis

All calculations were performed using the Statistical Package for Social Sciences software (version 17.0; SPSS, Inc.). The level of statistical significance was set at 0.05 for all tests.

Results

Mutated *JAK2* activates JAK-STAT pathway and up-regulates *HMGA2* expression

We hypothesized that *HMGA2* upregulation could be seen in cells with JAK-STAT signaling pathway activation, and chose to check its expressional status in MPN cells harboring either one of the two most common driver mutations (*JAK2* and *CALR*). As demonstrated in Figure 1A, there was an 8-fold increase in *Hmga2* levels in Ton.JAK2.V617F cells. The increment, however, was only around 2-fold in both Ba/F3 cells co-transduced with wild-type *MPL* and either type I (deletion) or type II (insertion) *CALR* mutants. Knowing that both mutated *JAK2* and *CALR* activated JAK-STAT signaling,[20-22] and considering the fact that a rise in *Hmga2* expression was more prominent in *JAK2*-mutated cells, we used mutated *JAK2* as our model of current investigation, but did not further explore *CALR*-mutated cells. To further validate the rise of *HMGA2* expression in *JAK2*-mutated cells, we screened four different human acute myeloid leukemia (AML) cell lines. Western blotting showed that *JAK2*V617F-carrying HEL and UKE-1 cells exhibited increased *JAK2* phosphorylation and enhanced *HMGA2* expression (Figure 1B). On the contrary, *HMGA2* expression was not increased in either *JAK2*-unmutated KG-1a or HL-60 cells. We next examined the signaling activity in cells with induced expression of JAK2. As demonstrated in Figure 1C, upon addition of doxycycline, Ton.JAK2.V617F cells exhibited enhanced JAK-STAT signaling as shown by increased phosphorylation of JAK2, STAT3, and STAT5. The level of HMGA2 protein was also increased significantly. On the other hand, the phosphorylation of JAK2 protein was rather weak in Ton.JAK2.WT cells. Although there was HMGA2 upregulation in these wild-type cells, the increment in HMGA2 expression was not as substantial as that seen in Ton.JAK2.V617F cells. Increased *Hmga2* transcripts could be observed at 2 days after induction of *JAK2*V617F expression (Figure 1D), and the levels of transcripts continued to rise after extended exposure to doxycycline in Ton.JAK2.V617F cells. These data demonstrate that mutated JAK2 could activate the JAK-STAT pathway and upregulate *Hmga2* expression in Ba/F3 cells.

HMGA2-overexpressing MPN patients exhibit unique clinical characteristics

To assess whether increased expression of *HMGA2* could be detected in clinical samples, we checked the levels of *HMGA2* transcripts in the granulocytes of 151 patients with MPN and 27 adult healthy controls. The cycle numbers (Ct) in all healthy individuals were greater than 40, indicating the expression of *HMGA2* was rather low in this population. We used the $2^{-\Delta\Delta}$CT method to calculate the relative *HMGA2* expression in our patients. Among the healthy individuals, the distribution of *HMGA2* transcript levels ranged between 0.02–3.0 times the mean level of the whole control population. Therefore, we defined the patients with a $2^{-\Delta\Delta}$CT value of *HMGA2* transcript greater than 3 as *HMGA2*(+), whereas the remainders were designated as *HMGA2*(-). Overall, 29 (19.2%) patients exhibited overexpression of *HMGA2* in their peripheral blood (PB) granulocytes. Table 1 demonstrates the clinical and laboratory characteristics of the 151 MPN patients subcategorized into the two groups. Patients whose granulocytes harbored increased *HMGA2* expression had a higher probability of carrying a driver mutation than those without, though the comparison was of borderline significance (93.1% *vs.* 77.0%, *P*=0.051). When taking only *JAK2*-mutated and triple negative (TN) patients into consideration, we had 22 (91.7%) *JAK2*-mutated and 2 (8.3%) TN patients in the *HMGA2*(+) group, and 78 (73.6%) and 28 (26.4%) in the *HMGA2*(-) group, respectively. The comparison regarding their difference was more significant (*P*=0.040, Table 1). Based on our *in vitro* data, it's plausible that *JAK2*V617F induced a higher degree of *Hmga2* upregulation (Figure 1A). Therefore, the difference in the frequencies of *JAK2* mutation between the *HMGA2*(+) and (-) groups became more prominent when we included only *JAK2* -mutated and TN patients as the denominator. Female patients constituted the main population of *HMGA2*(+) patients (65.5%), in contrast with that seen in the *HMGA2*(-) group (41.0%, *P*=0.017). Strikingly, compared to those without *HMGA2* overexpression, *HMGA2*-upregulated patients presented with a higher platelet count (789 ± 445 x 10^9/L *vs.* 576 ± 414 x 10^9L, *P*=0.016). This could be the result of a higher prevalent rate of *HMGA2* overexpression among ET patients (26.7%) as compared to that seen in PV patients (12.7%, *P*=0.044). Overall, *HMGA2*(+) MPN patients were more likely to suffer from major thrombotic events (34.5% *vs.* 15.6%, *P*=0.021). Consistently, Kaplan-Meier estimates showed that *HMGA2*-overexpressing MPN patients had a significantly inferior thrombosis-free survival (*P*=0.049, Figure 2). The cumulative incidences of thrombosis at 10 years were 31.0% and 13.9% in *HMGA2*(+) and

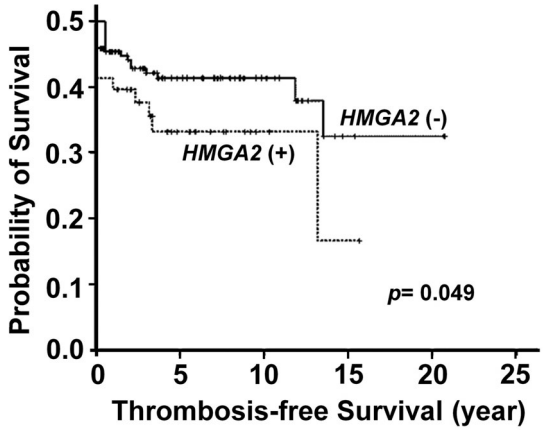

Figure 2. Impacts of *HMGA2* overexpression on clinical outcome of patients with MPN. Kaplan–Meier estimates of thrombosis-free survival (TFS) in 151 MPN patients stratified by the expression status of *HMGA2* showed those with upregulated *HMGA2* had significantly inferior TFS than those with lower expression (*P*=0.049, log-rank test).

HMGA2(-) patients, respectively. Coupled with the finding that *HMGA2*-overexpressing MPN patients were slightly older (65.7 ± 14.4 years old, *vs.* 59.5 ± 16.1, *P*=0.058), it's not surprising that *HMGA2*(+) ET/PV patients were more likely to carry high-risk disease (21/26; 80.8%), as defined by the European LeukemiaNet criteria,[15] than their *HMGA2*(-) counterparts (59/104; 56.7%; *P*=0.026). These data suggest that upregulation of *HMGA2* confers unique characteristics in patients with MPN, including a heightened probability of harboring either one of the 3 driver mutations, an increased propensity for the ET phenotype, and a higher likelihood of major thrombotic events.

Hmga2 overexpression confers *JAK2*V617F-mutated cells a survival advantage

To delineate if *Hmga2* overexpression altered cellular phenotypes, candidate cells were treated with either *Hmga2* siRNA or a scramble control to assess their respective characteristics (Figure 3). Figure 3A demonstrates the efficacy of *Hmga2* downregulation in both Ton.JAK2.WT and Ton.JAK2.V617F cells. Compared with a mock control or scrambled *siRNA*-treated control, Ton.JAK2.V617F cells treated with *Hmga2 siRNA* exhibited decreased viability by 50-60% (Figure 3B). The apoptosis assay revealed that the *siHmga2*-treated Ton.JAK2.V617F population had more cells in apoptosis (Figure 3C,D), a finding further confirmed by increased cleaved poly (ADP-ribose) polymerase (PARP) on western blotting (Figure 3E). Noticeably, the *Hmga2* knockdown efficiency was also confirmed by western blot analysis (Figure 3E). The data illustrate that *Hmga2* overexpression confers *JAK2*V617F-mutated cells with a survival advantage by rendering them more resistant to apoptotic death.

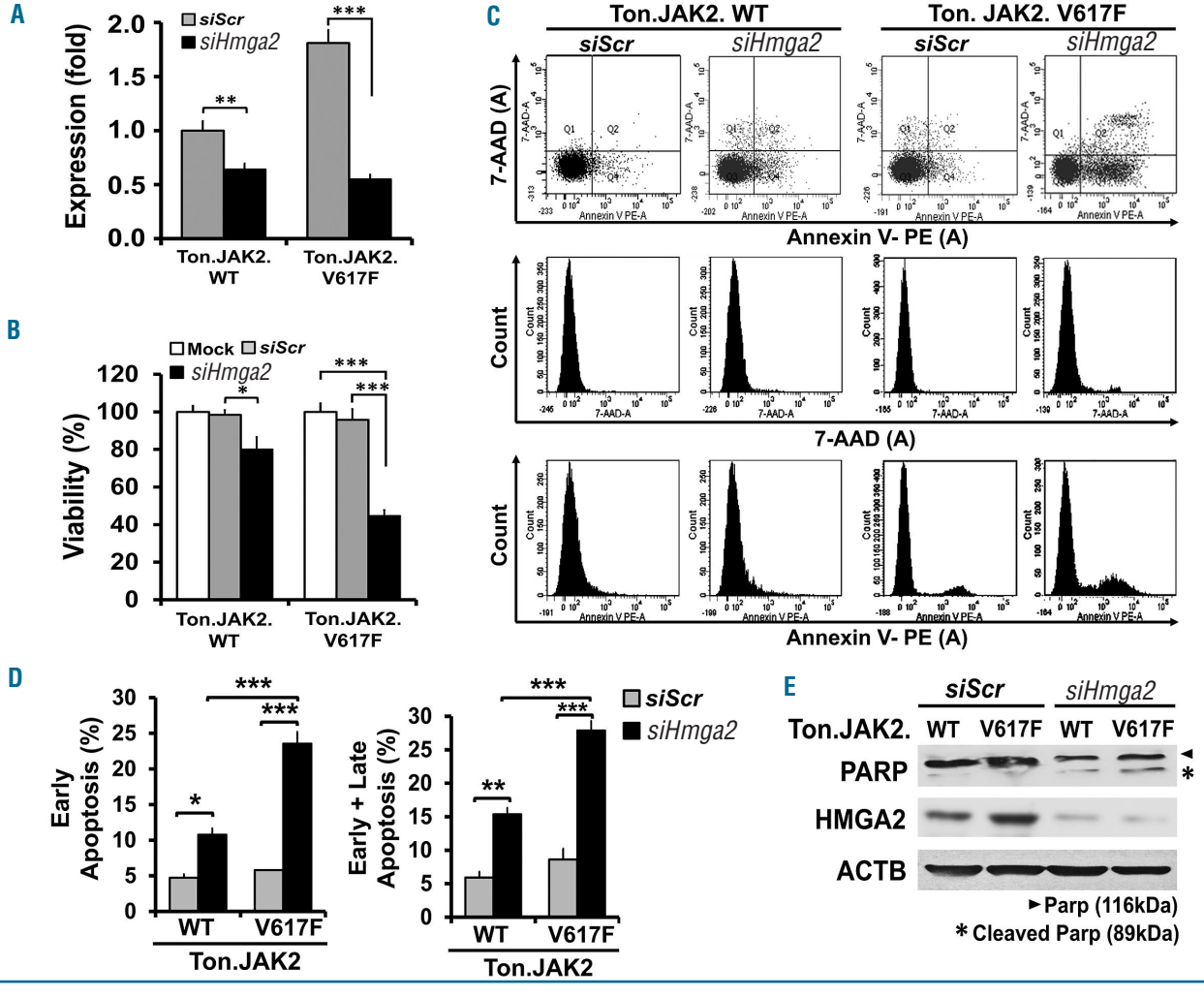

Figure 3. Effects of *Hmga2* downregulation in *JAK2*-mutated cells. Stable, inducible Ton.JAK2.WT and Ton.JAK2.V61F cells were pretreated with doxycycline (1 μg/mL) for 4 days and then transfected with either scrambled (*siScr*) or *Hmga2* siRNA (*siHmga2*). (A) Quantitative RT-PCR confirmed the knockdown efficiency. (*t*-test; ***P*<0.001; ****P*<0.0001). (B) Viability assay was assessed at 48 hours posttreatment with mock, *siScr* or *siHmga2* in Ton.JAK2.WT and Ton.JAK2.V61F cells. The error bars show the standard deviation of 3 independent experiments run in triplicate. Asterisks indicate t-test statistical significance (**P*<0.01; ****P*<0.0001). (C) Cell apoptotic rates were detected by flow cytometric analysis using PE conjugated-Annexin V and 7-AAD double staining. Cells positively stained with Annexin V only were defined as being in early apoptosis, whereas those with positive stains for both Annexin V and 7-AAD were considered as being late apoptotic cells. (D) Histogram of apoptotic percentages (**P*<0.01; ***P*<0.001; ****P*<0.0001, statistical significance by *t*-test). The data were collected from three individual experiments. (E) To detect the discrepancy of apoptotic rates between *siHmga2*- and *siScr*-treated cells, western blotting was performed to assess the amount of full and cleavage form of poly (ADP-ribose) polymerase (PARP). Arrowhead indicates the full-length of PARP protein (116kDa) and the asterisk represents the cleavage form of PARP (89kDa). WT: wild-type.

HMGA2 overexpression is associated with *let-7a* downregulation instead of chromosomal rearrangement around the 12q15 region

To clarify the mechanism that leads to abnormal *HMGA2* expression, we employed interphase FISH to detect potential cytogenetic abnormalities around this region, but found no abnormal rearrangement in all the cases evaluated (*data not shown*). We next assessed whether *let-7a* microRNA might play a role in the upregulation of *HMGA2* in MPN patients. As illustrated in Figure 4A, there was a modest inverse correlation between the expression levels of *let-7a* and *HMGA2* transcripts in the granulocytes of our patients (Pearson's correlation coefficient r=-0.291, P=0.0002). *In vitro* experiments disclosed that the gradual decline of the *let-7a* expression could be observed in Ton.JAK2.V617F cells upon the addition of doxycycline in a time-dependent manner (Figure 4B), as opposed to the progressive increment of *Hmga2* expression seen in similar assays (Figure 1D). To further validate whether the expression of HMGA2 could be regulated by *let-7a*, we first treated parental Ba/F3 cells with two different concentrations of a *let-7a* inhibitor. The result demonstrated that upon *let-7a* suppression, the expression of HMGA2 was increased in a dose-dependent manner at the protein (Figure 4C, upper panel) and mRNA (Figure 4C, lower panel) levels. Ectopic expression of *let-7a* in Ton.JAK2.V617F cells resulted in the downregulation of *Hmga2* mRNA (Figure 4D, right panel) and protein (Figure 4D, left panel), whereas cells treated with a *let-7a* inhibitor had increased HMGA2 expression. There was an inverse correlation between the expression levels of *Hmga2* and *let-7a* in those treated Ton.JAK2.V617F cells (r=-0.9281, P=0.002; Pearson's correlation). Overexpression of *let-7a* in Ton.JAK2.V617F cells also led to increased apoptosis, as shown by increased cleaved PARP and decreased phosphorylation of the pro-apoptotic protein Bcl-2-associated death promoter (BAD) (Figure 4D, left panel). Conversely, treatment with the *let-7a* inhibitor endued the Ton.JAK2.V617F cells with more resistance to apoptosis, as the cleaved PARP was reduced but the phosphorylated BAD was increased (Figure 4D). Importantly, the *let-7a* inhibitor significantly increased the cellular viability in both parental Ba/F3 cells and inducible Ton.JAK2.V617F cells, whereas overexpressed *let-7a* suppressed these *JAK2*-mutated cells' viability (Figure 4E). It was noteworthy that the levels of p-JAK2 were not significantly affect-

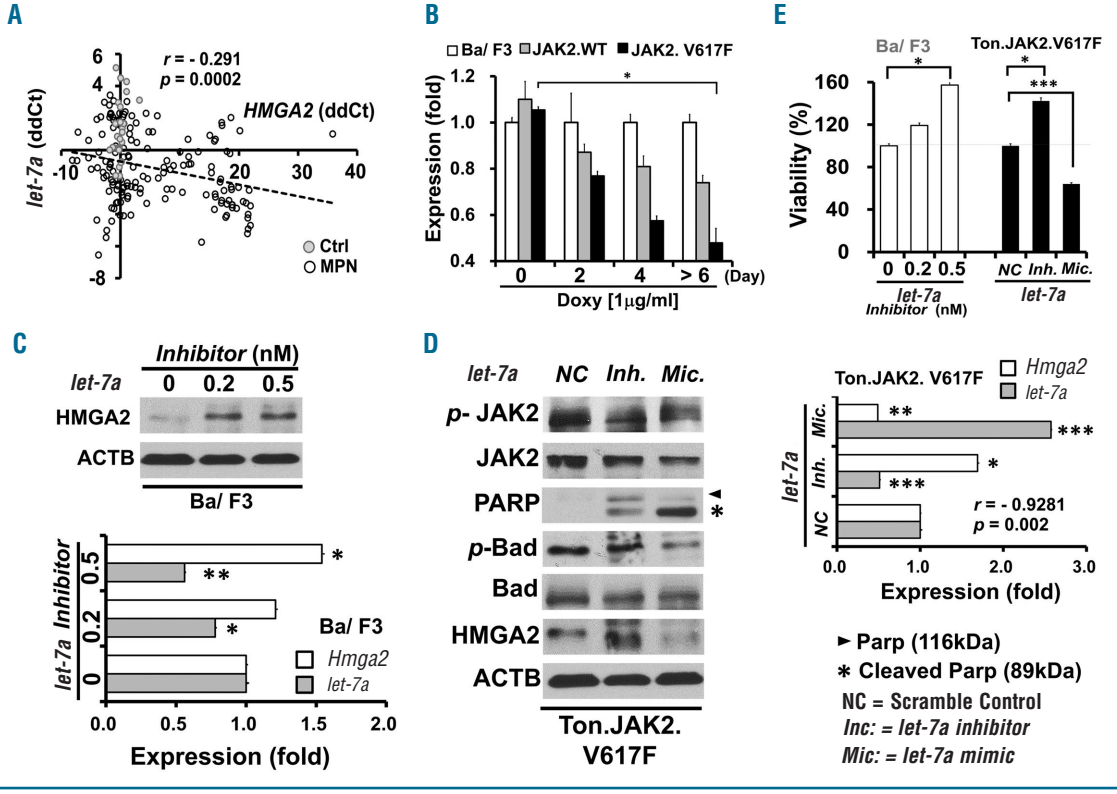

Figure 4. Correlation between the expression levels of *let-7a* and *HMGA2* transcripts in MPN cells. (A) Scatter plot illustrating the correlation between the expression levels of *let-7a* and *HMGA2* transcripts in the granulocytes of 151 patients with MPN (hollow circles; correlation coefficient r=-0.291, Pearson's correlation, P=0.0002). Note that data from healthy individuals (solid gray circles) mostly fell on the y-axis, and many of them were very close to the origin. Data are shown in ΔΔCT. (B) Quantitative RT-PCR was performed to detect the levels of *let-7a* miRNA transcripts in parental Ba/F3, Ton.JAK2.WT and Ton.JAK2.V61F cells after doxycycline treatment (1 μg/ml) for indicated periods of time. (C) Effects on a *let-7a* inhibitory oligo on the expression of *let-7a* and *Hmga2* in parental Ba/F3 cells. Two oligo concentrations (0.2 and 0.5 nM) were used for *let-7a* inhibition. All cells were harvested after 48 hours of transfection. Upper panel: western blot analysis of HMGA2; Lower panel: qRT-PCR analysis of *let-7a* and *Hmga2* transcript levels. (*t*-test; *P<0.01; **P<0.001). (D) Effects of *let-7a* inhibitory and mimic oligos on Ton.JAK2.V617F cells. The working concentration was 0.5 nM for both oligos. Right panel: qRT-PCR analysis of *let-7a* and *Hmga2* transcript levels. Note the inverse correlation between the expression levels of *let-7a* and *Hmga2* (correlation coefficient r=-0.9281, Pearson's correlation, P=0.002). Left panel: western blot analysis on the expression levels of JAK2, p-JAK2, PARP, cleaved PARP, BAD (Bcl-2-associated death promoter protein), p-BAD, and HMGA2. NC: scramble control; "-": treatment with a *let-7a* inhibitor; "+": treatment with a *let-7a* mimic. (E) The viability of parental Ba/F3 and inducible Ton.JAK2.V617F cells treated with indicated *let-7a* oligos. The error bars show the standard deviation of three independent experiments. Asterisks indicate statistical significance (*t*-test; *P<0.01; **P<0.001; ***P<0.0001).

ed by either ectopically expressed *let-7a* or *let-7a* inhibition (Figure 4D), indicating that alteration in the *let-7a-HMGA2* axis would not lead to a change in JAK-STAT signaling activity. These results imply that in MPN, *HMGA2* overexpression is mediated through *let-7a* downregulation, whereas chromosomal rearrangement around the 12q15 region might not play a potential role in this aspect. The data also further complement our earlier experiments showing that HMGA2 overexpression confers *JAK2*-mutated cells with a survival advantage (Figure 3).

Previous reports have demonstrated that LIN28A expression hinders the maturation of *let-7*.[23] Therefore, we also explored the potential roles of LIN28A in the *JAK2*V617F-mediated upregulation of *Hmga2*. We found that the expression levels of LIN28A in Ton.JAK2.V617F cells were also increased (*Online Supplementary Figure S2*). Nevertheless, when clinical samples were tested, we could not identify any association between the expression levels of *LIN28A* and *let-7a* (*Online Supplementary Figure S2C*).

Both hydroxyurea and pan-JAK inhibitor INC424 ameliorate JAK-STAT activation and alleviate HMGA2 upregulation

Hydroxyurea (HU) is considered an integral part in the treatment of patients with MPN.[15] To appraise the potential effects of HU on JAK-STAT signaling activity and HMGA2 expressional status, we first identified its estimated IC_{50} concentration (5.5μg/ml) in Ton.JAK2.V617F cells in a growth inhibition assay (Figure 5A). Subsequently, the cells were treated with hydroxyurea at

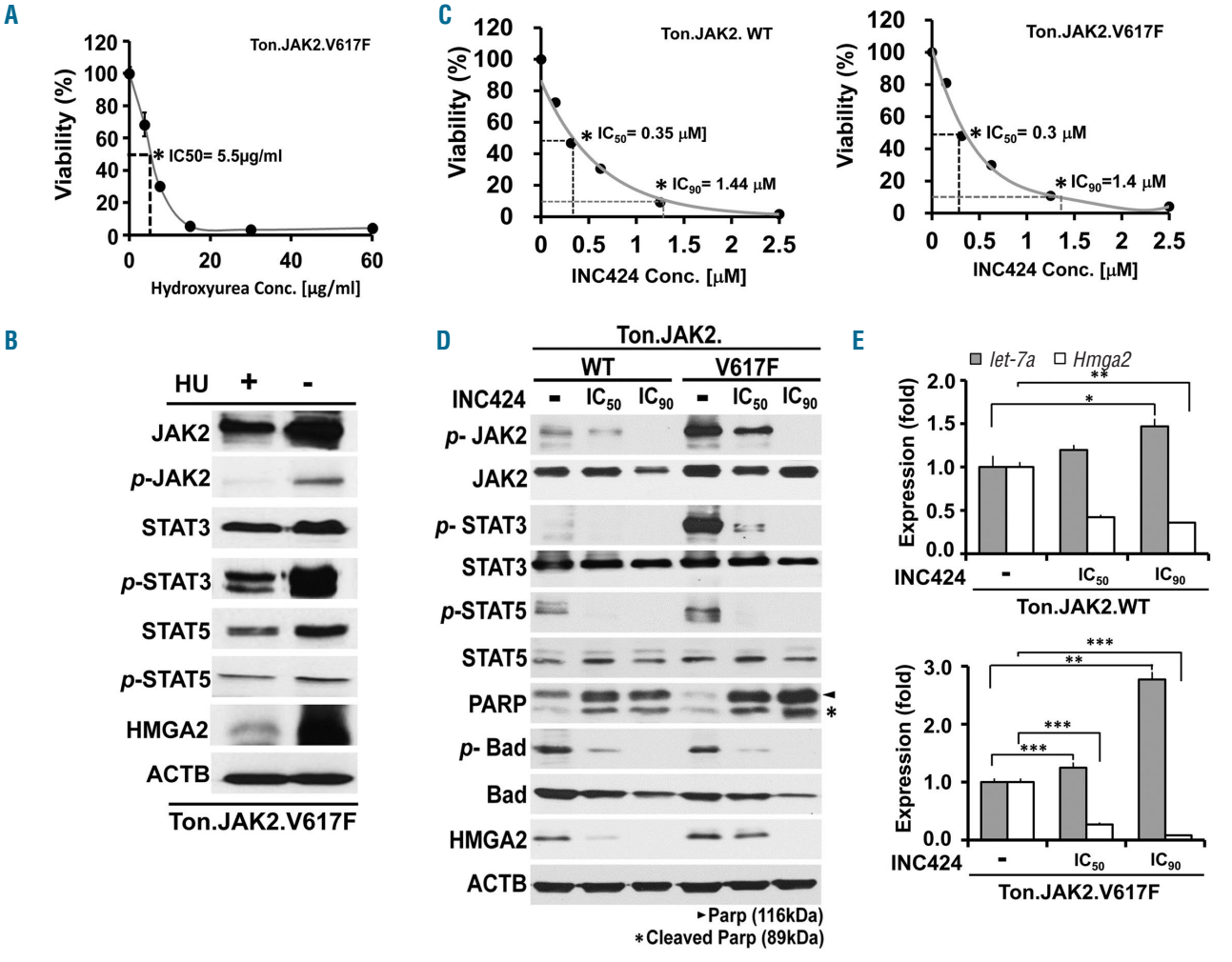

Figure 5. Influence of hydroxyurea and pan-JAK inhibitor INC424 on the phenotypes of MPN cells. Stable, inducible Ton.JAK2.V617F and Ton.JAK2.WT cells were treated with 1 μg/mL doxycycline for 6 days and then exposed to various concentrations of hydroxyurea or INC424 for 72 h. The drug concentrations that inhibited cell growth by 50% (IC_{50}) and 90% (IC_{90}) were determined by the XTT assay. (A) The viability of Ton.JAK2.V617F cells plotted against various concentrations of hydroxyurea. (B) Western blot analysis on various proteins of the Ton.JAK2.V617F cells treated with or without hydroxyurea at the concentration of 5.55 μg/ml (the IC_{50} concentration for HU in these cells) for 3 days. (C) The viability of Ton.JAK2.WT (left panel) and Ton.JAK2.V617F (right panel) cells plotted against various concentrations of INC424. (D) Western blot analysis on various proteins of the Ton.JAK2.WT and Ton.JAK2.V617F cells treated with or without INC424 at the concentration of either IC_{50} or IC_{90} for 3 days. (E) Effects of various concentrations of INC424 on the expression of *let-7a* and *Hmga2* in Ton.JAK2.WT and Ton.JAK2.V617F cells. Cells were harvested for qRT-PCR analysis at 3 days after treatment with the drug at indicated concentrations. The error bars show the standard deviation of three independent experiments. Asterisks indicate statistical significance (*t*-test; **P*<0.01; ***P*<0.001; ****P*<0.0001). WT: wild-type; HU: hydroxyurea; Conc: concentration.

the concentration for 3 days and then subjected to western blot analysis. As shown in Figure 5B, hydroxyurea abolished JAK2 phosphorylation almost completely, which in turn ameliorated downstream STAT3/STAT5 activity and significantly suppressed HMGA2 expression. To be more specific, we also selected INC424 (ruxolitinib, a pan-JAK inhibitor used in the treatment of MPN[24,25]) to assess the influence of JAK-STAT activity on the expression of HMGA2. Upon obtaining the IC_{50} and IC_{90} concentrations of INC424 in Ton.JAK2.WT and Ton.JAK2.V617F cells (Figure 5C), we treated the cells with the indicated drug concentrations for 3 days. Both sets of cells showed decreased JAK/STAT activity, abolished HMGA2 expression, and enhanced apoptosis in a dose-dependent manner (Figure 5D). The decreased *Hmga2* expression, as well as upregulated *let-7a* in INC424-treated cells, was also confirmed by qRT-PCR (Figure 5E). The results provide complementary evidence supporting a non-redundant role of JAK/STAT activation in *let-7a* inhibition and HMGA2 upregulation in MPN cells.

Discussion

In the study, we used Ba/F3 cells with conditional expression of *JAK2*V617F or WT *JAK2* as a working model to recapitulate the phenotypic comparison between *JAK2*-mutated and -unmutated MPN cells. We demonstrate that *JAK2*-mutated cells exhibit upregulation of HMGA2, and the expression of HMGA2 could be affected by the level of *let-7a* miRNA. HMGA2 overexpression confers *JAK2*-mutated cells with a survival advantage through inhibited apoptosis, and MPN patients harboring upregulated *HMGA2* show an increased propensity for ET phenotype as well as a higher likelihood of developing thrombosis.

The finding, in the study herein, that *HMGA2*-overexpressing MPN patients are more likely to belong to the ET subtype and have higher platelet counts is actually supported by several *in vivo* studies. Oguro *et al*. disclosed that overexpression of *Hmga2* in hematopoietic stem cells induced a myeloproliferative state with enhanced megakaryopoiesis in mice,[26] whereas Yang *et al*. similarly demonstrated that *Hmga2* significantly increased megakaryocytic colonies in the bone marrow of *JAK2*V617F mice.[27] Interestingly, Ikeda *et al*. showed that overexpression of *Hmga2* could lead to an increased level of *Jak2* transcripts and a rise in STAT3 phosphorylation.[13] As a result, the proliferation of hematopoietic stem cells was expanded. In the study herein, we further illustrated that HMGA2 knockdown in *JAK2*-mutated cells resulted in growth inhibition and a significant increase in apoptosis, a finding consistently seen in other cancer types.[28-30] Coupled with the fact that *Hmga2*-overexpressing BM cells have a growth advantage over control cells in mice competitive repopulation and serial BM transplantation models,[13] it is conceivable that the upregulated *HMGA2* "turns on" a state of proliferative hematopoiesis as well as inhibited apoptosis, and the predilection for megakaryocytic expansion[26,27] may account for a higher platelet count and ET phenotype in *HMGA2*-overexpressing patients.

However, the stronger association between *HMGA2* overexpression and ET subtype in the work herein contrasted with previous reports showing a higher prevalence of upregulated *HMGA2* in patients with PMF.[31,32] There are several possibilities that lead to such a discrepancy. Most likely, *HMGA2*-overexpressing ET and PMF might represent a continuum of disease entities at different stages, in which the overexpressed *HMGA2* collaborates with coexisting driver mutations or other pathognomonic mutations to cultivate the phenotypic expression. The argument can be supported by the fact that overexpression of *Hmga2* leads to both increased megakaryopoiesis and accelerated progression of myelofibrosis in animal models.[26,27] Secondly, patients from various studies have different ethnical and environmental backgrounds, which may contribute to the discrepancy in the *HMGA2*-overexpression rates in these reports, including ours. Lastly, the composition of the enrolled patients and the sample sizes across studies are different. Although currently available data suggest that overexpressed *HMGA2* might play a critical role in the pathogenesis of PMF,[12,13,26,27,31-34] in some studies the reproducibility of clinical data is hampered by either the low number of PMF patients evaluated or insufficient inclusion of PV and ET patients.[31-33] The Italian study[12] and ours enrolled the largest cohorts of patients, with the PMF population being the largest representative subgroup [n=88 (out of 158); 55.7%] in the former study. Supplemented by other smaller series, investigators from that study convincingly showed a high frequency of *HMGA2* overexpression in patients with PMF.[12] It is very likely that we had too few PMF patients (17 cases only) to make our estimation justifiable. On the other hand, our study enrolled by far the largest number of ET (n=67) and PV (n=63) patients for the assessment of *HMGA2* expression. We revealed that about one-fourth of ET patients harbored upregulated *HMGA2*, a finding mirrored by Harada-Shirado *et al*.[34] Although PMF patients comprised the major subgroup that showed upregulated *HMGA2* in the Italian study (as compared to ET and PV patients as well as normal controls), there was still a specific portion of ET patients who harbored overexpressed *HMGA2*.[12] Supported by the findings from these two studies,[12,34] our work provides further proof of evidence endorsing the notion that *HMGA2* overexpression not only plays an essential role in the pathogenesis of PMF, but also exerts specific effects on the phenotypic presentation of ET.

Although case reports suggested that chromosomal translocation involving the 12q14-15 region led to overexpression of *HMGA2* in patients with MPN,[35-38] our data nonetheless disclosed an essential role of *let-7a* miRNA regulation on the expression of HMGA2 in patients with MPN. These findings are in line with a recent report demonstrating that decreased *let-7* miRNA expression, instead of generating the loss of 3'-UTR of the *HMGA2* gene, is the major cause of dysregulated *HMGA2* mRNA expression in MPN.[34] The aberrancy in microRNA expression that leads to the genetic complexity of MPN is also supported by some other studies, as the reduction of a wide variety of miRNAs, including *let-7a*,[31] *miR-149*,[32] *miR-150*,[32] *let-7f*,[39] and *let-7g*[39] has been reported, and the former three are also associated with upregulated *HMGA2* mRNA in these reports.[31,32]

The correlation between aberrant *let-7a-HMGA2* activity and *JAK2*V617F mutation has been partially dissected by several investigators. Through transcriptome comparative microarray analysis, Guglielmelli *et al*. revealed that abnormal *HMGA2* expression in the granulocytes of patients with PMF was dependent on the presence of

*JAK2*V617F mutation.[12] Potential alteration in the expression of microRNAs, however, was not assessed in this study. Bruchova *et al*. performed gene expression profiling and found that *JAK2*V617F frequency was inversely correlated with *let-7a* expression in PV granulocytes.[31] Nevertheless, upregulated *HMGA2* was detected in PMF (but not in PV) patients, and no correlation between the expression levels of *let-7a* and *HMGA2* were identified.[31] With only 35 patients included, the study was probably hampered by small sample size. Although an inverse correlation between the expression levels of *let-7a* and *HMGA2* was found, Harada-Shirado *et al*. could not identify any potential relationship between *HMGA2* expressional status and *JAK2*V617F mutation.[34] Our study, enrolling significantly more patients, clearly demonstrates that *HMGA2*-overexpressing MPN patients are more likely to carry either one of the 3 driver mutations (Table 1). Coupled with our *in vitro* findings showing an apparent correlation between JAK-STAT activation and the *let-7a-HMGA2* axis, we comprehensively elaborated the dependence of aberrant *let-7a-HMGA2* axis activity on the presence of *JAK2*V617F mutation. A major flaw of our study might lie in the fact that we were unable to identify how mutant JAK2 regulates the *let-7a-HMGA2* axis activity. Further exploratory work would be important to delineate the missing link between them.

In conclusion, we have demonstrated that enhanced *Hmga2* expression could be seen in Ba/F3 cells with enforced *JAK2*V617F expression. *HMGA2*-overexpressing patients exhibit a trend of a higher likelihood of carrying one of the 3 MPN-relevant driver mutations. *In vitro* data confirm that downregulation of *let-7* miRNA plays an essential role in the dysregulated expression of *Hmga2*; a result supplemented by the inverse correlation between the expression levels of *let-7* and *HMGA2* in clinical samples. Strikingly, expression of HMGA2 confers *JAK2*-mutated cells with a survival advantage and endues MPN patients with a unique clinical phenotype. Our findings suggest that, in a subset of MPN patients, the *let-7-HMGA2* axis plays a prominent role in the pathogenesis of the disease that leads to unique clinical phenotypes.

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