miR-28 is a thrombopoietin receptor targeting microRNA detected in a fraction of myeloproliferative neoplasm patient platelets

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Abstract

BCR-ABL negative myeloproliferative neoplasms (MPN) (Polycythemia Vera, Essential Thrombocythemia, Primary Myelofibrosis) are malignant diseases arising from a multipotent hematopoietic progenitor, frequently altered by JAK2 V617F or other JAK/STAT activating mutations. The thrombopoietin receptor (TpoR, MPL) is one of the major dimeric cytokine receptors that utilize JAK2 in the myeloid lineage, and was found to be down-modulated in certain MPN patients. We searched for negative regulators of MPL expression. Here we report that miR-28 targets the 3' untranslated (3'UTR) region of MPL, inhibiting its translation, as well as other proteins potentially involved in megakaryocyte differentiation, such as E2F6. Expression of miR-28 in CD34-derived megakaryocytes inhibited terminal differentiation. miR-28 was found to be overexpressed in platelets of a fraction of MPN patients, while it was expressed at constant low levels in platelets from healthy individuals. Constitutive activation of STAT5 leading to autonomous growth of hematopoietic cell lines was associated with increased miR-28 expression. We discuss how down-modulating MPL and other targets of miR-28, and of related miR-708 and miR-151, could contribute to MPN pathogenicity.
Introduction

The BCR-ABL negative myeloproliferative neoplasms (MPN), Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) are malignant diseases arising from a mutant multipotent hematopoietic stem cell (HSC)\(^{1,2}\), that are associated with constitutively active JAK-STAT signaling. The \textit{JAK2} V617F mutation is present in 95% of PV, 50% of ET and PMF\(^{3,4,5,6}\). \textit{JAK2} exon 12 mutations are found in a minority of PV patients\(^7\). 5% of PMF and 1% of ET harbor thrombopoietin receptor (MPL, TpoR) W515 mutations\(^8,9\), which constitutively activate JAK2 signaling\(^10\).

Moliterno \textit{et al.} have reported diminished platelet MPL expression in MPNs\(^11\) and an inverse correlation between \textit{JAK2} V617F allele burden and MPL expression, although down-modulation of MPL was observed also in \textit{JAK2} V617F-negative patients\(^12\). This suggests that mechanisms that limit the expression, surface localization and function of MPL might operate during the establishment of MPNs.

We searched for putative microRNAs\(^13\) that target the 3'UTR of the MPL mRNA. We found that miR-28 is an inhibitor of MPL translation, which is also the case for two close relatives of miR-28, miR-151 and miR-708. We identified several miR-28 targets, besides MPL, such as E2F6, a transcription factor involved in the control of proliferation and apoptosis, and the MAP-kinase MAPK1/ERK2. We then detected induction of miR-28 in cell lines transformed by \textit{JAK2} V617F or activated MPL mutants. We have also investigated levels of expression of miR-28 in platelets from healthy subjects and MPN patients.
Materials and methods

Cell lines, plasmid/luciferase constructs and reagents

Human erythroleukemia (HEL), JAK2-deficient cell γ2A, UT-7, Mo-7e and Ba/F3 cells were maintained as described \(^{14,15,16,17,18}\). The UKE-1 cell line was a kind gift of Dr. Walter Fiedler, University Hospital Eppendorf, Hamburg, Germany. Translational inhibition by microRNAs was measured using the psi-CHECK™-2 reporter vector (Promega). Briefly, 3’UTR specific primers extended with adaptor sequences corresponding either to a NotI or XhoI site were used in a PCR reaction. PCR products were cloned in the TOPO vector (Invitrogen) and positive clones were digested by NotI and XhoI and ligated in the digested psi-CHECK™-2 vector. After sequencing, the microRNA target sites were mutated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following manufacturer instructions. These plasmid constructs (0.8 µg) were transfected with 40 nM pre-miR™ (Ambion) in γ2A cells using Lipofectamine (Invitrogen). Luciferase activities were assayed 48 h after transfection using the Dual-Luciferase® Reporter Assay System (Promega).

Pri-miRs were amplified with pri-miR-28 primers (28s GGCAACATCTAAATATGGCTTG; 28a TGAGGTAGGCAGTATTAGCTCTGA), pri-miR-708 primers (708s GAAACCTAACCCCCATGGTT; 708a TAGAGGGTCCTCAGGTGGTG) and pri-miR-151 primers (151s AGCTGAGCCTGGTGCTAGTC; 151a ATTCAGTGCCTGGGTGACTC), cloned in Topo TA vector (Invitrogen), digested by EcoRI and ligated in pMEGIX-IRES-GFP. All constructs were verified by sequencing. Retroviral transduction were performed as described \(^{19}\). The dominant negative STAT5 (STAT5AΔ749) (originally described by Dr. T. Kitamura) was cloned in pREX-IRES-CD2 and used for Ba/F3 TpoR JAK2 V617F or HEL cells electroporations. JAK2 inhibitors were from Calbiochem (JAK inhibitor 1), AstraZeneca (AZD1480), Sigma (AG490) and SYNthesis med chem (TG101209).
Patient samples
Platelets were isolated from MPN patients of Hôpital Henri Mondor, Paris and from St Luc Hospital, Brussels. Blood samples were obtained with approval of all participating institutions' Ethical Committees and informed consent in accordance with the Declaration of Helsinki. The JAK2 V617F and MPL W515 status was determined by TaqMan PCR and sequencing as described\(^\text{12}\).

Real-Time quantitative PCR
Total RNA, including miRNA and other small RNA molecules, was isolated using TRIZol (Invitrogen) and subsequently purified with the miRNeasy kit (Qiagen) following manufacturer’s instructions. Stem-loop reverse transcription and quantitative real-time PCR have been performed as described\(^\text{20}\), using the following RT-Primers: GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TCA AT (RT-miR28); GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA AAA ATA TG (RT-U6); Forward primers: CGTCAAGGAGCTCACAGTCTA (FW-miR28); AAATTCGTGAAGCGTTCCAT (FW-U6); the universal reverse primer GTGCAGGGTCCGAGGT (RC); and the miR-28 probe 5'(6-FAM)-ACTGGATACGACCTCAA-3'(BHQ1). Quantities of miR-28 are normalized to U6 levels in each sample and are depicted as relative quantities to the normalized miR-28 levels of a control cell line, i.e. UT7, Mo7e, Ba/F3, or HEL, that is indicated on the y-axis.

Expression analysis of the LPP gene was performed by real-time PCR with the following primers: LPPs GTGCAATGTGTGTTCCAAGC; LPPa TGGCATAATAGGCTCCTTGC. Data were normalized to beta-actin amplified with the following primers: ACTBs CCTGGCACCCAGCACAAT; ACTBa GGGCCGGACTCGTCATACT.
5’-Rapid Amplification of cDNA Ends (5’RACE-PCR)

Amplification of 5’ ends of LPP mRNAs was performed using the SMART RACE cDNA Amplification Kit protocol (Clontech) and LPP gene specific primers: LPP-GSP1 TGGCAGCCAAGATGGGTGAGACATT and LPP-GSP2 CCTCTGATGAGCGTCCAGTGGAACA. The PCR products were analyzed on a 1.2 % agarose gel. PCR bands were excised and purified with the QIAquick gel extraction kit (Qiagen). Purified DNAs were cloned with the TOPO TA cloning kit (Invitrogen) and eight positive clones from each transformation were sequenced.

Chromatin immunoprecipitation

Chromatin cross-linking, cell lysis and DNA shearing were performed as described 21. Immunoprecipitation was performed with the OneDay chromatin immunoprecipitation (ChIP) kit (Diagenode), as recommended by the manufacturer. Antibodies for immunoprecipitation of STAT5B and RNApolII were purchased from Millipore (#06-969) and Diagenode (#AC-055-100) respectively. Primers for ChIP qPCR analysis were designed with the primer3 software (http://frodo.wi.mit.edu/primer3/) and tested for amplification efficiency and specificity on genomic DNA. Validated primers were used for real-time PCR on purified DNA after ChIP. The primers used for qPCR are ProAs TTGAGCACAGGACAGAGGAA; ProAa TTTTAGCCCTGAGCCTTGAA ; Pro1s TTGCATTCTGGATGGTCTCA ; Pro1a AGGCTGGAGGATGTCAGAAA ; 3’s AAAAGCAAACCTTGCCTGAA ; 3’a TGAAGAGAGCCAATGAACGA. Primers for actin were identical to those described at Real Time quantitative PCR section, and the PCR product does not contain any putative STAT-binding sites.
**Retroviral transductions**

Retroviruses produced after BOSC packaging cells transfections were used to infect Ba/F3 cells as described \(^\text{19}\). GFP positive cells were sorted 72 hours after infection. Cell numbers were recorded with a Coulter cell counter.

**CD34 cultures and megakaryocyte differentiation in liquid culture**

Liquid cultures using human CD34+ cells were retrovirally transduced with bicistronic viruses coding for miR-28, empty vector (pMegix) and GFP. Cells were maintained in serum-free medium containing Tpo and proplatelet counting was performed on GFP/CD41 double positive megakaryocytes, as described \(^\text{22}\). Megakaryocyte micrographs (x40 objective) were taken with a Nikon TE300 microscope (Tokyo, Japan) at 37 °C in serum-free medium containing Tpo. Photos were taken with an AxioCamMR camera and processed with AxioVisionRel.4.6, CarlZeiss (Göttingen, Germany)

**Western blotting**

MPL protein levels were evaluated in Mo7e cell using the Anti-TpoR/c-Mpl antibody (Millipore, 06-944). Cells were harvested, washed in cold PBS and resuspended in cold NP40 Lysis Buffer (50mM Tris-Cl pH8, 150 mM NaCl, 1% NP40, 0.1 mM EDTA, 10 mM NaF, 1 mM Na\(_3\)VO\(_4\), 1 mM PMSF, 1 mM DTT). Upon incubation on ice for 30 min with occasional vortexing, lysates were centrifuged for 20 min at 20,000 g and 4°C. Supernatants were collected and protein quantification were performed with the BCA™ Protein Assay Kit (Pierce). Protein extracts (50 µg) were loaded on NuPage 4-12% Bis-Tris gels (Invitrogen) and Western blot analysis was performed using a 1:1,000 MPL antibody and 1:10,000 dilution anti-rabbit-horseradish peroxidase antibodies (GE Healthcare, UK). The membranes were stripped and reprobed with anti-actin beta (monoclonal anti-ACTB, Sigma-Aldrich).
Results

The MPL 3’UTR is a target of miR-28

Five potential microRNA targets were identified with the miranda (http://www.microrna.org) and miRBase (http://microrna.sanger.ac.uk) programs in the 3’UTR MPL mRNA, starting at nucleotides 2,157; 2,531; 2,711; 2,836 and 3,524. The MPL 3’UTR was cloned downstream the luciferase coding region of a reporter vector and used to test the ability of microRNAs to inhibit translation. None of the let-7 family (let-7a, c, d, e, f and miR-98) pre-miRs (precursor of microRNA) inhibited translation (Fig. 1A). miR-28 inhibited the luciferase activity by ~30%, as compared to the activity of a control microRNA, that does not recognize any potential target. Since miR-28 could potentially bind to two distinct sites (starting at nucleotides 2,157 and 3,524, respectively), we performed mutagenesis of each of these sites (Supplementary Fig. 1). We observed that mutagenesis of nucleotides 3,530-3,542 relieved the inhibition of luciferase activity by miR-28, while mutagenesis of the region encompassing nucleotides 2,168-2,174 did not. Thus, miR-28 inhibited luciferase expression by binding to MPL 3’UTR nucleotides 3,524-3,545.

To assess whether miR-28 inhibits translation of endogenous MPL, we transduced the human Mo7e cell line with a bicistronic retroviral vector expressing miR-28 along with the GFP. Mo7e cells express endogenous MPL and can proliferate in medium supplemented with MPL ligand thrombopoietin (Tpo). Cells infected with the empty retrovirus (pMegix) or miR-28 expressing retrovirus (miR-28) were sorted for equivalent GFP levels. We observed an inhibition of MPL translation by miR-28 (Fig. 1B). Importantly, expression of miR-28 did not reduce the level of endogenous mRNA for MPL in Mo7e or in HEL cells (not shown).
miR-28 and sequence related microRNAs target the MPL mRNA and other mRNAs coding for proteins involved in proliferation and apoptosis

We identified two microRNAs related to miR-28 (paralogs) with the BLASTN program found on the miRBase web site (http://microrna.sanger.ac.uk/). miR-151 has 80% sequence identity with miR-28, and miR-708 is 68% identical to miR-28 and 71% with miR-151. Mo7e cells were infected with bicistronic retroviral vectors coding for miR-28, 708 or 151 along with the GFP. After sorting for equivalent GFP levels, we monitored MPL protein levels (Fig. 2A) and Tpo induced cell proliferation (Fig. 2B). miR-28 and miR-708 were both inhibiting MPL translation by about 20%, while miR-151 inhibited it by 50%. miR-151 was also more potent that miR-28 or -708 for inhibition of Tpo mediated cell proliferation. Thus, miR-28 and sequence related miRs inhibit Tpo induced proliferation by MPL down-regulation.

Furthermore, as expected from the functional data, all the miRs targeted the MPL 3'UTR for translational inhibition (Fig. 2C). The combination of miR-28 with miR-151 and miR-708 did not result in significantly synergic inhibition of luciferase activity, consistent with the notion that they all target the same sequence in the 3'UTR of MPL (Fig. 2C).

To identify targets recognized by miR-28 other than MPL, we used the miRBase target prediction program and the BLASTn program against the Refseq mRNA database. Fourteen targets displaying $\Delta G \leq -26$ kcal/mol (the miR-28:MPL 3’UTR predicted free energy) were retained for further analysis. We next selected mRNAs expressed in blood cell lineages (http://genome.ucsc.edu). Five targets were selected: N4BP1, OTUB1, TEX261, MAPK1 and E2F6. We tested using the psi-CHECK™-2 luciferase reporter assay the ability of these microRNAs to inhibit translation of the five targets (Fig. 2D). We observed an up to 80% inhibition of N4BP1 (NEDD4 binding protein 1), a regulator of the E3 Ubiquitin Ligase-
Itch\textsuperscript{23}, by all three miRs. OTUB1 (Otubain1), an inhibitor of GRAIL (gene related to anergy in lymphocytes), and TEX261 (testis expressed 261) were both inhibited by miR-28 and -708 by about 40%. MAPK1 (mitogen-activated protein kinase 1 Transcript variant 1 also known as Extracellular regulated kinase 2-ERK2) was inhibited by miR-28 by 40%. E2F6 (E2F transcription factor 6) was inhibited by miR-28 and -151 by 40% and 20%, respectively. E2F6 is a negative regulator of E2F1 transcription factor involved in cell-cycle regulation and apoptosis\textsuperscript{24}. MPL and the last two targets are potentially important for megakaryocyte differentiation, since E2F1 was shown to impair megakaryocyte differentiation\textsuperscript{25} and MAPK1 was shown to be required for megakaryocyte differentiation\textsuperscript{26}.

**Effects of miR-28 expression on megakaryocyte proplatelet formation**

To assess the functional consequences of miR-28 expression on megakaryocyte proplatelet formation, CD34+ hematopoietic progenitors were infected with bicistronic retroviruses co-expressing miR-28 and GFP, or empty vector and GFP, and then maintained in medium containing Tpo, as described\textsuperscript{22}. Megakaryocytes were examined after 14 days of culture. The number of proplatelet-bearing CD41/GFP positive megakaryocytes was significantly inhibited (>50%) by miR-28 expression (Figs. 2E and F). Although we did not co-express JAK2 V617F and miR-28 in these experiments to determine the effect of miR-28 in megakaryocytes expressing JAK2 V617F, our data suggest that miR-28 exerts a negative role on megakaryocyte differentiation. It remains to be determined whether this effect is linked directly to the down-modulation of MPL or to the other targets we identified, such as E2F6 or MAPK1.
Constitutive STAT5 activation in transformed hematopoietic cells is associated with miR-28 expression

We found that the HEL cell line, which has been derived from a patient that relapsed with erythroleukemia after being treated for Hodgkin's lymphoma \(^{14}\), and the UKE-1 cell line derived from a patient with ET, that transformed to acute leukemia \(^{27}\) expressed high levels of miR-28 (Fig. 3A); UKE-1 cells are homozygous for the JAK2 V617F mutation \(^{6}\), while HEL cells have 8 copies of the JAK2 locus and are homozygous for JAK2 V617F \(^{28}\) (Fig. 3A). Both HEL and UKE-1 cells express much higher levels of miR-28 than UT-7 cells (Fig. 3A), which is a human megakaryocyte leukemia cell line that expresses very high levels of wild type JAK2.

To test whether miR-28 expression was indeed linked to JAK2 V617F expression, we infected the parental UT-7 cell line, negative for JAK2 V617F and miR-28 expression, with bicistronic retroviral vectors expressing either JAK2 WT or JAK2 V617F together with GFP. After sorting for equivalent GFP levels, JAK2 V617F mRNA represented only about 12% (11.88 ± 0.42) of the total JAK2 mRNA cell content. A similar increase (about 12%) in the level of JAK2 WT mRNA was observed in UT-7 cells engineered to overexpress JAK2 WT (UT-7 JAK2 WT) (Supplementary Fig. 4). This limited overexpression of JAK2 V617F or JAK2 WT that we could attain is likely due to the fact that UT-7 cells express very high endogenous JAK2 levels (i.e. 5 fold higher than in UKE-1 cells). Nevertheless, miR-28 was expressed at 3-fold higher levels in JAK2 V617F UT-7 cell line, when compared to the JAK2 WT cell line (Fig. 3B). These data indicate that expression of the constitutive active JAK2 V617F leads to expression of miR-28.
Next, we stably transduced the erythropoietin receptor (EpoR) or MPL (TpoR) in Ba/F3 cells together with JAK2 WT or JAK2 V617F and sorted cells for receptor expression and for equivalent levels of JAK2 WT and JAK2 V617F. Ba/F3 cells are IL3-dependent murine proB cells. Neither IL-3, Epo or Tpo stimulations increased miR-28 expression in parental Ba/F3, Ba/F3-EpoR or Ba/F3-TpoR, respectively. Similarly, Ba/F3 EpoR JAK2 WT and Ba/F3 TpoR JAK2WT cell lines did not respond by miR-28 induction after cytokine stimulation. In contrast, the Ba/F3 EpoR JAK2 V617F and Ba/F3 TpoR JAK2 V617F cell lines, expressed 6 and 8 fold more miR-28, and these levels were significantly increased in a dose-dependent manner (not shown) by further cytokine stimulation (Fig. 3C). Thus, miR-28 is specifically induced by the constitutive JAK2 V617F signaling.

The Ba/F3 cells co-expressing TpoR and JAK2 V617F were then selected for autonomous growth. As expected, miR-28 level further increased when the JAK2 V617F constitutive activity was enhanced (by 4-fold, not shown). The JAK2 inhibitors: AG490, JAK Inhibitor I, AZD1480 and TG101209 were all potent to reduce miR-28 expression (Fig. 4A), suggesting that miR-28 expression is dependent on the catalytic activity of JAK2 V617F.

Similarly, when Ba/F3 were transformed to autonomous growth by expression of TpoR W515A/L/K or TpoR Δ5 (TpoR deleted in the juxtamembrane RWQFP motif that includes W515), which are constitutively active mutants of MPL, we observed an increase in miR-28 expression (Fig. 4B).

Another means to induce cytokine-independent growth of Ba/F3 cells via constitutive STAT5 activation is to express the Bcr-Abl oncogene; indeed Bcr-Abl also increased miR-28 levels. Since JAK2 V617F, TpoR W515 mutants and Bcr-Abl are all activating STAT5, we then
tested a constitutively active mutant of STAT5, STAT5 1*6, which contains two point mutations, H299R and S711F, that lead to strong constitutive activation. Cells expressing STAT5 1*6 displayed a 30-fold increase in miR-28 levels, indicating that STAT5 constitutive activation is linked to induction of miR-28 expression. Finally, we electroporated Ba/F3 TpoR JAK2 V617F cells, selected for autonomous growth, with a vector expressing a dominant negative mutant of STAT5 (STAT5DN) together with the CD2 surface marker. After monitoring CD2 surface expression by FACS, we observed an inhibition of miR-28 expression in STAT5DN expressing cells compared to control cells electroporated with an empty vector (Fig. 4B). A similar decrease in miR-28 expression could be noted when the STAT5DN was electroporated in HEL cells (Fig. 4C). These results demonstrate that miR-28 expression is dependent on constitutive STAT5 activity induced by JAK2 V617F.

**The constitutive STAT5 activity in transformed cells is inducing miR-28 host gene transcription**

miR-28 is encoded by the sixth intron of the LIM domain lipoma-preferred partner (LPP) gene. This gene was first isolated as part of a fusion protein created by chromosomal translocations in lipomas and certain pulmonary chondroid hamartomas. Chromosomal rearrangements were also discovered with the mixed lineage leukemia (MLL) gene in secondary acute leukemia (sAML). The breakpoint t(3;11)(q28;q23) occurred in MLL and LPP intron 8. The physiologic function of LPP is unknown. Since miR-28 is expressed at 35-fold higher levels in HEL than in UT-7 cells (Fig. 5A), we tested whether the LPP gene was expressed at higher levels in HEL versus UT-7 cells, in the scenario that miR-28 expression is induced via expression of its host gene, and not driven by an internal promoter. Indeed, LPP levels are dramatically increased in HEL, when compared to UT-7 cells (Fig. 5A). In order to localize the promoter of the LPP gene and therefore of miR-28 in HEL cells,
we performed 5’Rapid Amplification of cDNA ends (RACE) PCR on HEL and UT-7 cells (Fig. 5B). While we detected the expected 200 bp band in UT-7 cells (according to the UCSC Genome Browser), a larger band (around 300-400 bp) was detected in HEL cells. After cloning of PCR products and sequencing of multiple clones, this 300-400 bp band was found to contain the LPP exon 2 and two alternative exons (A and B) of 94 bp and 119 bp located 59 kb and 4.7 kb upstream the regular transcription start site, respectively, amounting to 315 bp (Fig. 5B). Thus, LPP transcription appears to involve, at least in HEL cells, an alternative upstream promoter, when compared to the normal LPP transcription in UT-7 cells. Furthermore, expressing JAK2 V617F in UT-7 cells also induced an increase in LPP mRNA levels, in parallel with induction of miR-28 expression (Fig. 5B).

We next searched for STAT binding sites, defined by the TTC(N)₃GAA consensus, and that are conserved between the mouse and human LPP. No STAT binding site was identified near the promoter 1 (Prom 1) transcription start site, in agreement with lack of miR-28 (or LPP) induction in cells transiently stimulated with cytokines. A number of STAT5 potential sites were predicted along LPP (Supplementary Fig. 2). Detailed experiments are required to test the relevance of each site for transcription of LPP. One of those STAT5-binding sites was close (2 kb) to the alternative transcription site detected in HEL cells. We asked whether the alternative promoter (Prom A) (Fig. 5C) might be a binding target for the constitutive active STAT5 via this site. Direct binding of STAT5B to LPP promoter sites - Prom 1 or Prom A - (Fig. 5C) was then assessed in UT-7 JAK2 V617F, in UT-7 JAK2 WT and in HEL cells by STAT5B chromatin immunoprecipitations (ChIP) and then by PCR amplification of either Prom 1/ Prom A, or of actin as a negative control, since no STAT5 binding sites were contained in the amplified actin sequence. We detected enriched STAT5B binding to Prom A site in HEL compared to UT-7 cells (Fig. 5D). The difference in STAT5B binding between
UT-7 JAK2 V617F and UT-7 JAK2 WT cells was below significance, although the former expressed higher miR-28 levels. This might be due to long miR half-life and the very low levels of JAK2 V617F that could be transduced in UT7 cells. In order to seek for transcriptional activity, RNA pol II ChIP were performed on both promoter sites. We detected an increased RNA pol II binding to promoter A site in JAK2 V617F expressing cell lines compared to JAK2 WT expressing cell lines (Fig. 5D), suggesting that STAT5 activates the transcription of Prom A site.

Expression of miR-28 in platelets from MPN patients

In order to evaluate whether increased miR-28 expression might be detected in MPN patients, small-size RNA was isolated from platelets of 18 healthy donors and 53 MPNs patients (8 PV, 29 ET and 16 PMF) were extracted and monitored for miR-28 expression levels by Stem-Loop qRT-PCR (Figure 6). miR-28 was expressed in three out of six JAK2 V617F-positive PV patients, one out of 9 JAK2 V617F-positive ET, 9 out of 20 JAK2 WT ET, one out of six PMF JAK2 WT, and two out of four MPL W515 mutant-positive PMF (Fig. 6A); the MPL W515 mutations also lead to constitutive JAK/STAT activation 8-10. Taken together, miR-28 is expressed in platelets of about 30% of MPN patients. When we evaluated miR-151 expression in patients’ platelets, only two patients, namely ET JAK2 V617F patient-4 and PMF MPL W515L patient-2 displayed significant upregulated levels. We were not able to evaluate miR-708 levels in patients’ platelets due to cross-reactivity of the tested probes.

The mean miR-28 platelet level in MPNs was 11 fold greater than in controls (p=0.0137) (Fig. 6B). In order to evaluate whether a relationship can be established between expression of JAK2 V617F and of miR-28 in platelets, we set-up a genotyping test for the quantitation of JAK2 V617F and JAK2 WT at the mRNA/cDNA level (as platelets do not have genomic
DNA), using the same TaqMan® probes used for JAK2 V617F detection at the genomic DNA level. We observed a good correlation between JAK2 V617F mRNA in MPNs granulocytes and the allelic ratio measured from granulocytes DNA ($R^2=0.9607$) (Supplementary Fig. 4). Importantly, the JAK2 V617F levels (ratio to total V617F+WT JAK2) at the mRNA level were similar in granulocytes and platelets from the same patient (Supplementary Fig. 4). This test allowed us to measure levels of JAK2 V617F mRNA in platelets from MPN patients.

miR-28 levels were compared between two platelet groups, one expressing <50% and the other >50% JAK2 V617F. The >50% JAK2 V617F group displayed a 34-fold increase (p=0.0034) in miR-28 expression level, indicating a possible correlation between JAK2 V617F allele burden and miR-28 expression. Importantly, miR-28 is expressed in platelets, but not in granulocytes (Fig. 6C). Since megakaryocytes are major players in the pathogenicity of MPNs $^{33,34}$, we suggest that these data might be relevant for further understanding of molecular anomalies of this lineage in MPNs.

Separately from the analysis of JAK2 V617F-positive patients, we could observe that 9 out of 20 ET JAK2 WT patients overexpressed miR-28 (Figure 6A). Since the ET JAK2 WT group is known to have higher platelet counts $^{35}$, it is possible that miR-28 represents a marker of megakaryocyte proliferation. In order to establish this correlation, a larger study is necessary.

Next we examined the MPL protein levels for 21 (5 PV, 12 ET, 4 PMF) of the 53 MPN patients and asked whether a correlation could be found between high miR-28 levels and MPL down-modulation (Supplementary Figure 3). Of the five PV patients examined, five had down-modulation of MPL, but only four had high miR-28; PV patient 6 had an allele burden of JAK2 V617F of 93%, down-modulation of MPL and low miR-28 (see Discussion). All four MPL W515L/K PMF patients exhibited strong down-modulation of MPL; two of the
patients had high miR-28 levels, and a third (PMF MPL W515L patient 2) had overexpressed miR-151 levels. Hereby we therefore report that MPL W515 mutation also is associated with MPL down-modulation.

For ET patients, seven out of twelve did not present platelet MPL protein down-modulation (Supplementary Figure 3). Two ET patients were positive for JAK2 V617F, ET patients 8 and 7, with allele burdens of 26% and 35%, respectively. No miR-28 or MPL down-modulation could be detected. However, in ET patients normal and mutated clones co-exist, and the levels of miR-28 and MPL that we can detect represent averages of normal and mutated clones. For ET patients that did not exhibit JAK2 V617F, it is impossible to know the allele burden of the putative "other mutations"; interestingly three such ET patients had high miR-28 and no MPL down-modulation. Whether this simply reflects the inability of miR-28 to down-modulate platelet MPL in ET or the presence of a mix of normal and high miR-28/weak MPL down-modulation is not known. The nature of the other events responsible for JAK2 V617F/negative ET might also affect the induction and effects of miR-28.
Discussion

Our major observations are that: i) miR-28 and two related miRs (miR-708 and miR-151) target the 3'UTR of MPL for down-modulation, and ii) a fraction of MPN patients, especially PV, and ET patients wild type for JAK2, overexpress miR-28 in their platelets. Since the JAK2 WT ET group is known to exhibit higher platelet counts, miR-28 might represent a marker of megakaryocyte hyperproliferation.

We identified a number of targets of miR-28. Perhaps the most relevant, after MPL, for megakaryocyte differentiation is E2F6, a transcription factor belonging to the E2F family; E2F6 actually represses E2F-responsive genes, by inhibiting E2F1, which was previously shown to inhibit megakaryocyte terminal differentiation. The mitogen-activated protein kinase 1 (MAPK1, ERK2: extracellular signal-regulated kinase 2) is inhibited by miR-28, but not by miR-708 or miR-151, and this might be relevant as the Ras/MAPK pathway is important for megakaryocyte differentiation. CD34+ derived megakaryocyte differentiation assays suggest that miR-28 plays a negative role on differentiation of precursors to proplatelet bearing megakaryocytes. We propose that miR-28 induction might be part of negative feedback mechanisms invoked by megakaryocyte proliferation of MPNs.

In cytokine-dependent and leukemia cell lines cytokine stimulation and activation of STAT5 did not suffice to induce miR-28 expression; this is mirrored by absence of STAT5-binding sites near the described promoter of LPP, the host gene for miR-28. Additional events are therefore required for miR-28 induction, besides transient STAT5 activation. Constitutive STAT5 activation in Ba/F3 cells is associated with increased miR-28 expression; this effect might reflect cooperation between permanently activated STAT5 and yet to be described
transcription factors. In HEL cells, induction of the LPP/miR-28 gene occurs via an upstream alternative promoter that contains a STAT5-binding site. Chromatin changes around the LPP alternative promoter might occur in transformed cells, but more experiments are required to elucidate LPP induction in transformed cells.

A fraction of patients with MPNs overexpress miR-28 in their platelets. This was true for PV (with JAK2 V617F), PMF with MPL W515L/K and surprisingly for ET with JAK2 WT. It would be expected that patients with JAK2 V617F harbor high miR-28 levels in platelets. How can we explain that PV patient 6 does not overexpress miR-28, although the JAK2 V617F allele burden was 93%? As stated before, miR-28 induction might require additional events besides constitutive STAT5 activation, and might depend on the state of megakaryocyte proliferation. Another possibility would be that the CpG islands of the alternative LPP promoter are methylated in these patient. Finally, constitutive active STAT5 might be diverted from the LPP gene by other proteins.

We could perform the analysis of platelet MPN levels for 21 (5 PV, 12 ET and 4 PMF) of the 53 MPN patients in order to test whether the MPL down-modulation is correlated with high miR-28 levels. PV and PMF patients exhibited MPL protein down-regulation in platelets (Supplementary Fig. 3), including the one PV patient (patient 6) that expressed low levels of miR-28. All four MPL W515 mutant PMF patients exhibited down-modulated MPL, but only two had high miR-28 levels, while the third (MPL W515L patient 2) overexpressed miR-151 (not shown). No PV or PMF patient with high miR-28 showed high MPL protein. Thus, most patients with PV and PMF had MPL down-modulation, but not all had high miR-28. In the ET group, three patients (9, 13 and 16) exhibited high miR-28 and high MPL protein. Since in ET normal and disease/mutated clones co-exist, high overexpression of
miR-28 could remain detectable, while a low (20%) decrease in MPL translation that would be induced by miR-28 might be masked by the normal clones. These results indicate that miR-28 can contribute to but is not the sole mechanisms responsible for MPL down-modulation in platelets. This is expected since MPL has a long half-life \(^{40, 41}\), and MPL down-modulation by enhanced internalization, ubiquitinylation and degradation can be detected in cells expressing JAK2 V617F (Pecquet et al., unpublished observations), a process that is mimicked by Tpo-induced TpoR down-modulation \(^{40}\).

The existence of microRNAs in platelets has recently been documented \(^{42}\). No miR-28 upregulation was detected in MPN platelets in a microRNA profiling study \(^{43}\), since the microarray did not contain any miR-28 probe. Another microRNA-profiling study of PMF granulocytes did not detect miR-28 increases \(^{44}\). In agreement with this result, we show that miR-28 is specifically expressed in platelets, but not in granulocytes from the same patient. Currently, we attempt to identify the precise stage during the megakaryocytic differentiation where miR-28 induction occurs, given that, normally, miR-28 should not be expressed past the pre-megakaryocyte stage \(^{45}\).

In conclusion, we found that miR-28 is a potent inhibitor of MPL translation and of several other proteins potentially important for megakaryocyte differentiation, such as E2F6 and ERK2. We suggest that expression of miR-28 might play important roles in the pathogenicity of MPNs, either as part of negative feedbacks of myeloproliferation or as regulator of disease phenotype.
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Author contribution

Contribution: M.G. designed the research, performed experiments and wrote paper, C.P. produced key cell lines, S.B. performed experiments, L.K., A.F., S.G. and W.V. selected and provided patient samples and wrote paper, S.N.C. designed the research and wrote the paper. The authors declare no competing financial interests.
References


Figure legends

Figure 1: miR-28 recognizes the MPL 3’UTR and inhibits its translation.
(A) γ2A cells were cotransfected either with 0.8 µg MPL 3’UTR luciferase reporter (gray histograms) or MPL 3’UTR luciferase reporter mutated for the site of interest (black histograms) and either 40 nM Pre-miR-28, Pre-let-7a, 7c, 7d, 7e, 7f, Pre-miR-98 or Pre-miR-control. Values represent the mean luciferase activity ± SD of three independent experiments relative to Pre-miR-control transfected cells. The 1,680 nt MPL 3’UTR sequence was cloned after the stop codon of the luciferase and is represented with the mRNA coordinates of the putative miR binding sites (gray boxes). (B) Mo7e cells were transduced with a bicistronic retroviral vector expressing miR-28 together with the GFP. After sorting for equivalent GFP levels, cell lines expressing either the empty vector (pMegix) or the miR-28 expressing vector (miR-28) were subjected to Western blotting for MPL protein levels. The histogram reports MPL relative bands intensities measured on Western blot.

Figure 2: miR-28 and closely related miR-151 and miR-708 inhibit Tpo-dependent proliferation of Mo7e cells and target mRNAs coding for proteins involved in proliferation and apoptosis.
(A) Mo7e cell lines transduced with a bicistronic retrovirus (pMegix) expressing the GFP protein along with indicated microRNAs. Western blot analyses for MPL and β-Actin (ACTB) protein levels were performed. Normalized MPL protein levels are indicated below. (B) Mo7e cell lines expressing miR-28, miR-708 or miR-151 were grown in the presence of indicated amounts of Tpo or GM-CSF (Granulocyte Macrophage Colony Stimulating Factor)
for 4 days. Cell proliferation is represented as fold increase compared to cell lines grown without cytokines. Shown are averages of triplicates ± S.D. of one representative experiment out of three. (C) 3’UTRs (gray bars) of indicated mRNAs or 3’UTRs mutated for miR-28 target sites (black bars) were cloned after the stop codon of the renilia luciferase in the psi-CHECK™-2 reporter vector. These luciferase reporter vectors were co-transfected with miR expressing vectors. (D and E) CD34+ hematopoietic progenitors were infected with pMegix bicistronic retrovirus co-expressing miR-28 with the GFP or the control retrovirus expressing only the GFP. After 14 days of culture, proplatelet-bearing CD41/GFP-positive megakaryocytes were counted. Shown in E are numbers of proplatelet-bearing CD41/GFP positive megakaryocytes (average of triplicates + S.D. of one representative experiment).

**Figure 3: Constitutive signaling by JAK2 V617F induces miR-28 expression.**

(A) miR-28 relative expression was measured in the HEL and UKE-1 cell lines, which are homozygous for JAK2 V617F and in UT-7 parental cell line, which is negative for JAK2 V617F. The JAK2 V617F and WT expression levels are reported below. (B) miR-28 relative levels were assessed in UT-7 parental cell line transduced with a bicistronic retrovirus expressing the GFP along with either JAK2 WT or JAK2 V617F and sorted for equivalent GFP levels. Asterisks indicates p<0.01. (C) Ba/F3 cells were stimulated with 20 ng/ml of IL-3 for 3 h. Ba/F3 EpoR and Ba/F3 TpoR cells co-expressing equivalent JAK2 WT or JAK2 V617F levels (sorted cells) were stimulated for 3 h with 20 units/ml Epo or 20 ng/ml Tpo respectively.
Figure 4: miR-28 is induced by JAK2 V617F, MPL W515 mutants, Bcr/Abl and STAT5 1*6.

Ba/F3 cell lines expressing indicated proteins were tested for miR-28 relative expression. 
(A) JAK2 V617F human TpoR-Ba/F3 cells selected for autonomous growth were treated with the JAK2 inhibitors: AG490 (10 µM); JAK Inhibitor I (0.5 µM); AZD1480 (3 µM), TG101209 (3 µM) or vehicle only (-) for 24 h (*: p<0.05; **: p<0.001). Absence of toxicity was demonstrated by Trypan blue staining. (B) Ba/F3 cells expressing the indicated human TpoR mutants, Bcr/Abl or STAT5 1*6 were selected for autonomous growth in absence of cytokines and tested for miR-28 expression (*: p<0.0001). (C) The indicated cell lines were electroporated either with an empty expression vector expressing the CD2 surface marker (pREX-CD2), or the same bicistronic expression vector co-expressing the STAT5DN (dominant negative) mutant with the CD2 surface marker. Electroporation levels were verified by FACS and the miR-28 expression was quantified by stem-loop qRT-PCR. The presented results are representative of three independent experiments.

Figure 5: LPP, the miR-28 host gene, is over-expressed in HEL cells through the transcriptional activation of an upstream alternative promoter bound by STAT5.

(A) Relative expression of the LPP (LIM domain lipoma-preferred partner) transcript and miR-28 in the indicated cell lines. The LPP level in UT-7 parental and UT-7 JAK2 WT cell lines have been arbitrarily set at 1. (B) 5’-Rapid Amplification of cDNA Ends (RACE)-PCR: a nested PCR on cDNA integrating an adaptor sequence at the 5’end of the LPP transcript amplified a 200 bp band in UT-7 and a larger 300-400 bp band in HEL cell lines. After sequencing, the 216 bp band amplified from UT-7 contains exon 1 and 2 of the LPP gene. The 300-400 bp band amplified from HEL contains two alternative exons (A and B) and exon
2 of the LPP gene. (C) A schematic view of the human LPP gene (LIM domain lipoma-preferred partner) represented with its 11 exons (black boxes), the two alternative exons (A and B) and the pri-miR-28 stem loop sequence in intron 6. The two identified promoters are indicated by arrows and labeled as Prom A (alternative) and Prom 1 (normal start site). (D) Quantitative PCR on indicated targets after chromatin immunoprecipitation (ChIP) relative to the starting DNA quantity before immunoprecipitation (% INPUT ± SD). Gray and black histograms represent ChIP performed on JAK2 WT and V617F UT-7 cells or UT-7 and HEL cells, respectively. White histograms are negative controls. The actin gene amplification (ACTB) was used a negative control for STAT5B binding. Quantitative PCR for RNA pol II ChIP were normalized against a non transcribed region located outside (3’) of the LPP gene.

**Figure 6: Detection of miR-28 overexpression in platelets from MPN patients.**

(A) miR-28 relative levels are depicted in phe positive control that is represented by Mo7e cells transduced by the pMegix vector overexpressing miR-28 (± SD quantification done in triplicate). Ctrl: control platelets from healthy volunteers; PV: Polycythemia vera; ET: Essential thrombocythemia; PMF: Primary Myelofibrosis. Status for JAK2 or MPL mutations is indicated below. (B) Relative miR-28 levels in control and MPN platelets. Mean miR-28 levels are represented on graphs (p = 0.0137; Mann-Whitney test). Positive JAK2 V617F patients were grouped accordingly to their allele burden (<50% or >50% JAK2 V617F) and examined for miR-28 levels (p = 0.0034 Mann-Whitney test). (C) miR-28 relative levels in platelets and granulocytes of the same patient. Patient numbers and normalization of relative miR-28 quantity are the same as in (A).
Figure 4

A

% miR28 expression

AG490  |  Jak inhibitor I  |  AZD1480  |  TG01209

Ba/F3 MPL JAK2 V617F

B

miR-28/U6 (rel. BaF/3)

Ba/F3  |  MPL  |  MPL Δ5  |  MPL W515A  |  MPL W515L  |  MPL W515K  |  Bcr/Abl  |  STAT5 1*6

C

BaF/3 MPL JAK2 V617F

miR-28/U6 (rel. NT)

- STAT5DN

HEL

- STAT5DN
miR-28 is a thrombopoietin receptor targeting microRNA detected in a fraction of myeloproliferative neoplasm patient platelets

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