Increased phospho-mTOR expression in megakaryocytic cells derived from CD34+ progenitors of essential thrombocythaemia and myelofibrosis patients

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that forms different multiprotein complexes with diverse subunit compositions, downstream substrates and biological effects (Ma & Blenis, 2009). As mTOR is a key regulator of cell growth and metabolism, its improper activation has been previously linked to carcinogenesis (Meric-Bernstam & Gonzalez-Angulo, 2009).

Polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) are *BCR-ABL1*-negative myeloproliferative neoplasms (MPNs). ET is characterized by megakaryocytic bone marrow hyperplasia and a sustained elevation of platelet count with a tendency for thrombosis and haemorrhage (Levine & Gilliland, 2008). PMF can arise '*de novo*' or evolve from PV or ET. In PMF, the abnormal proliferation of an aberrant clone eventually leads to the replacement of the normal bone marrow with connective tissue fibres (Tefferi & Vainchenker, 2011). As the molecular culprit(s) of these disorders still need(s) to be elucidated, current treatment options are not curative and have yet to produce clear benefits in terms of increasing overall survival (Tefferi & Vainchenker, 2011).

Mammalian target of rapamycin is a pivotal downstream target of the thrombopoietin receptor (Drayer *et al*, 2006). Thus, it is not surprising that several studies have previously demonstrated critical contributions of mTOR signalling in the proliferation and differentiation of normal megakaryocyte (MK) cultures (Drayer *et al*, 2006; Raslova *et al*, 2006). However, the role of mTOR in the megakaryopoiesis of ET and PMF cells remains to be investigated. As thrombocytosis is a common clinical feature of both ET and PMF, we wanted to establish whether mTOR activation was involved in the increased platelet counts observed in these MPNs.

To this end, CD34-positive (CD34+) progenitors were employed to reproduce the different stages of human MK differentiation that generate morphologically and functionally mature platelets (Guerriero *et al*, 1995; Deutsch & Tomer, 2006). After acquiring informed consent, human CD34+ cells were isolated from healthy donors, ET and PMF patients using the midi-MACS immunomagnetic separation system (Miltenyi Biotec, Auburn, CA, USA) obtaining a >90% pure cell population. Megakaryocytic differentiation was then induced by growing these cells for 14 d in serum-free medium in the presence of bovine serum albumin, insulin, human transferrin, human low-density lipoprotein, and 100 ng/ml thrombopoietin (PeproTech, London, UK).

The expression of the CD34 and CD61 surface markers was evaluated by flow cytometry, collecting cells at different time-points (days 0, 3, 12) and using a fluorescein isothiocyanate-labelled anti-CD34 antibody and an anti-CD61 antibody bound to phycoerythrin (Miltenyi Biotec). A gradual decrease in the CD34 signal associated with increased expression of the MK-specific CD61 marker confirmed the purity of the initial population and its progressive megakaryocytic differentiation (Figure S1A). Morphological observations after May–Grunwald Giemsa staining (Sigma, St Louis, MO, USA) correlated with the flow cytometry data, showing large polynucleated MK cells after 12 d of culture (Figure S1B).

A unilineage system was subsequently employed to establish if MK cells isolated from patients with ET or PMF expressed higher levels of phosphorylated mTOR (p-mTOR) when compared to healthy individuals. We initially performed an immunofluorescence (IF) analysis in which cells were stained with an anti-p-mTOR (recognizing serine 2448) antibody (Cell Signaling, Danvers, MA, USA) after 3 and 12 d of culture. While we found no differences in mTOR phosphorylation after 3 d (Fig 1A, top panels), p-mTOR was increased in MKs from ET and PMF patients obtained after 12 d of culture (Fig 1A, bottom panels), but not in cells derived from healthy donors. To quantify the qualitative differences detected by IF, we analysed mTOR phosphorylation by flow cytometry, evaluating the x-fold increase in median fluorescence intensity over the isotype control (D'Asaro *et al*, 2010). This analysis confirmed higher p-mTOR expression in ET and PMF-derived MKs generated after 12 d of culture (Fig 1B).

To further confirm these data, we performed an anti-p-mTOR immunohistochemical analysis on 35 bone marrow specimens derived from patients diagnosed with ET ($n = 14$) or PMF ($n = 21$). Three micrometre-thick sections were incubated with the same anti-p-mTOR antibody employed for the IF analyses. We found higher p-mTOR expression in 34 of 35 bone marrow samples as compared to the mostly negative staining observed in healthy individuals (Fig 2).

The present study provides the first comparison of p-mTOR expression in MK cells derived from the CD34+ progenitors of healthy donors, ET and PMF patients. Recent evidence suggests that targeting mTOR with the oral inhibitor everolimus may be clinically useful for patients

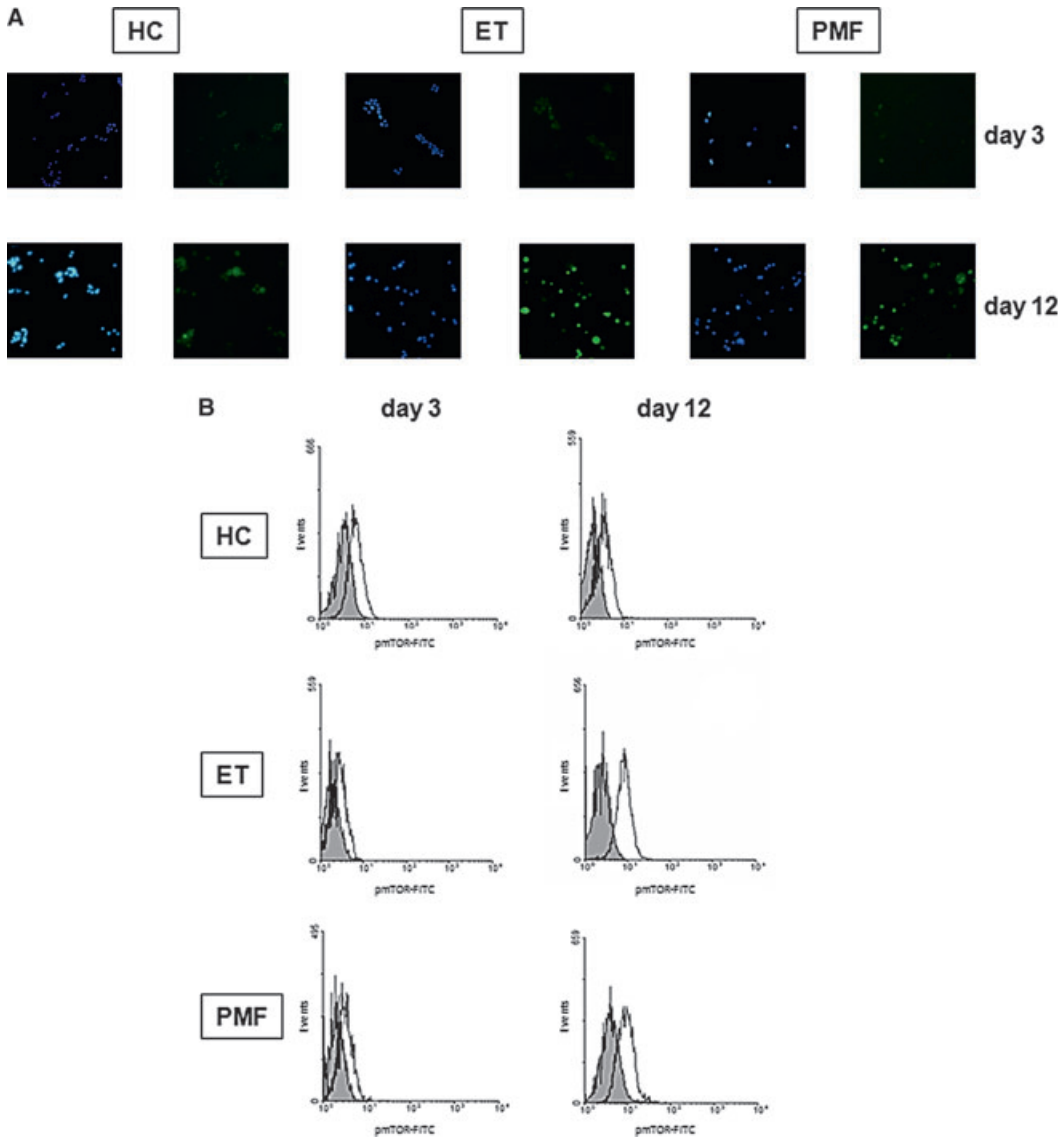

Fig 1. Increased p-mTOR expression during MK differentiation of CD34+ cells isolated from ET and PMF patients. (A) Immunofluorescence analysis of p-mTOR expression in CD34+ progenitors of healthy controls (HC), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) patients during MK differentiation (days 3 and 12). Cells were harvested at the specified times, fixed and stained with an anti p-mTOR (green) antibody. DNA (blue) was coloured using 4′6-diamidino-2-phenylindole (Sigma). (B) mTOR phosphorylation analysis by flow cytometry. Cells, collected after the indicated days of culture, were stained for p-mTOR and subsequently incubated with a fluorescein isothocyanate (FITC)-conjugated secondary antibody. Experiments were analysed using the FACSAria II flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

developing PMF. Indeed, 25% of PMF patients successfully normalized their platelet counts while on everolimus (Guglielmelli *et al*, 2011). Our findings provide a mechanistic rationale for these results, demonstrating increased mTOR phosphorylation during the megakaryocytic differentiation of ET and PMF progenitors, and confirming a role for mTOR targeting in individuals affected by these MPNs.

Acknowledgments

We thank Maria Rita Pulvirenti and Sandro Barbuscia for their technical assistance and Gaetano Insolia for image rendering.

Authors Contribution

LuV and DM designed the study, performed most of the experiments, analysed the results and wrote a draft of the manuscript; SB performed all flow cytometry experiments and contributed to data analysis; CC and EA performed immunohistochemistry and data analysis; FS and MC provided all primary samples; LoV provided formalin-fixed paraffin-embedded BM specimens; FDR, MG and RDM critically revised the manuscript; PV contributed to the experimental design and wrote the final version of the manuscript that was approved by all Authors.

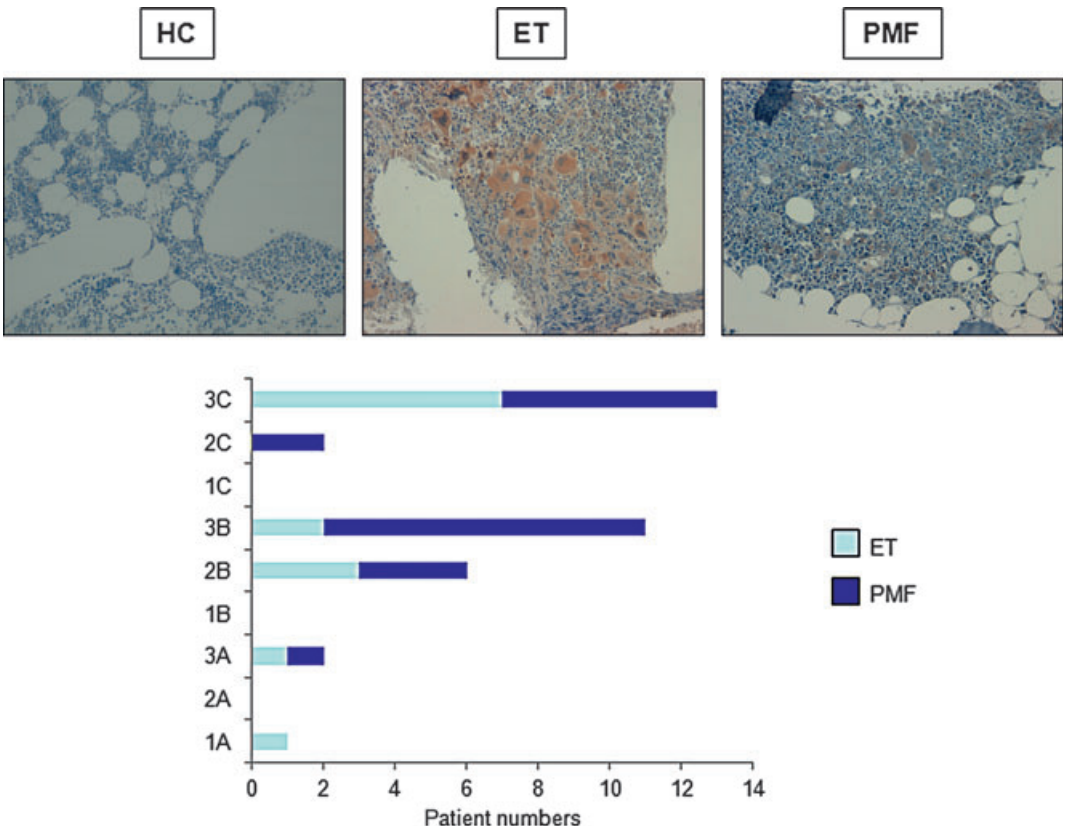

Fig 2. Increased p-mTOR expression in immunohistochemical analyses performed on bone marrow samples of ET and PMF patients. Immunohistochemical analysis of p-mTOR expression in formalin-fixed paraffin-embedded bone marrow samples from healthy controls (HC) and essential thrombocythaemia (ET, $n = 14$) and primary myelofibrosis (PMF, $n = 21$) patients. Slides were incubated with the previously indicated p-mTOR antibody and counterstained with haematoxylin. Each slide was scored by two pathologists blinded to the patient's characteristics. Staining was defined as cytoplasmic localization above background level and scored as follows: A = weak; B = moderate; C = strong. The absence of cytoplasmic staining was reported as negative. Percentage of stained cells was assessed as follows: 1 = 1–25%; 2 = 25–50%; 3 = >50%.

Luisa Vicari[1]
Daniela Martinetti[1]
Simona Buccheri[1]
Cristina Colarossi[2]
Eleonora Aiello[2]
Fabio Stagno[3,4]
Loredana Villari[4]
Maide Cavalli[3,4]
Francesco Di Raimondo[3,4]
Massimo Gulisano[1]
Ruggero De Maria[5]
Paolo Vigneri[3]
[1]IOM Ricerca, [2]Department of Experimental Oncology, Mediterranean Institute of Oncology (IOM), [3]Department of Clinical and Molecular Bio-Medicine, University of Catania, Catania, Italy, [4]Division of Haematology, Ferrarotto Hospital, Catania, and [5]Regina Elena National Cancer Institute, 00144, Rome, Italy
E-mail: pvigneri@libero.it

Keywords: mTOR, megakaryopoiesis, unilineage System, essential thrombocythaemia, myelofibrosis

First published online 24 August 2012
doi: 10.1111/j.1365-2141.2012.09246.x

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Flow-cytometric and morphological evaluation of MK cultures derived from CD34+ progenitors of healthy donors, ET or MF patients.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

D'Asaro, M., La Mendola, C., Di Liberto, D., Orlando, V., Todaro, M., Spina, M., Guggino, G., Meraviglia, S., Caccamo, N., Messina, A., Salerno, A., Di Raimondo, F., Vigneri, P., Stassi, G., Fournie, J.J. & Dieli, F. (2010) V gamma 9V delta 2 T lymphocytes efficiently recognize and kill zoledronate-sensitized, imatinib-sensitive, and imatinib-resistant chronic myelogenous leukemia cells. *Journal of Immunology*, **184**, 3260–3268.

Deutsch, V.R. & Tomer, A. (2006) Megakaryocyte development and platelet production. *British Journal of Haematology*, **134**, 453–466.

Drayer, A.L., Olthof, S.G. & Vellenga, E. (2006) Mammalian target of rapamycin is required for thrombopoietin-induced proliferation of megakaryocyte progenitors. *Stem Cells*, **24**, 105–114.

Guerriero, R., Testa, U., Gabbianelli, M., Mattia, G., Montesoro, E., Macioce, G., Pace, A., Ziegler, B., Hassan, H.J. & Peschle, C. (1995) Unilineage megakaryocytic proliferation and differentiation of purified hematopoietic progenitors in serum-free liquid culture. *Blood*, **86**, 3725–3736.

Guglielmelli, P., Barosi, G., Rambaldi, A., Marchioli, R., Masciulli, A., Tozzi, L., Biamonte, F., Bartalucci, N., Gattoni, E., Lupo, M.L., Finazzi, G., Pancrazzi, A., Antonioli, E., Susini, M.C., Pieri, L., Malevolti, E., Usala, E., Occhini, U., Grossi, A., Caglio, S., Paratore, S., Bosi, A., Barbui, T. & Vannucchi, A.M. on behalf of the AIRC-Gruppo Italiano Malattie Mieloproliferative (AGIMM) investigators. (2011) Safety and efficacy of everolimus, a mTOR inhibitor, as single agent in a phase 1/2 study in patients with myelofibrosis. *Blood*, **118**, 2069–2076.

Levine, R.L. & Gilliland, D.G. (2008) Myeloproliferative disorders. *Blood*, **112**, 2190–2198.

Ma, X.M. & Blenis, J. (2009) Molecular mechanisms of mTOR-mediated translational control. *Nature Reviews Molecular Cell Biology*, **10**, 307–318.

Meric-Bernstam, F. & Gonzalez-Angulo, A.M. (2009) Targeting the mTOR signaling network for cancer therapy. *Journal of Clinical Oncology*, **27**, 2278–2287.

Raslova, H., Baccini, V., Loussaief, L., Comba, B., Larghero, J., Debili, N. & Vainchenker, W. (2006) Mammalian target of rapamycin (mTOR) regulates both proliferation of megakaryocyte progenitors and late stages of megakaryocyte differentiation. *Blood*, **107**, 2303–2310.

Tefferi, A. & Vainchenker, W. (2011) Myeloproliferative neoplasms: molecular pathophysiology, essential clinical understanding, and treatment strategies. *Journal of Clinical Oncology*, **29**, 573–582.

Sub-optimal inhibition of thrombus formation *ex vivo* by aspirin in patients with primary thrombocythaemia

Given that patients with primary thrombocythaemia (PT) are prone to thrombosis, first-line therapy with aspirin, an irreversible cyclooxygenase (COX)-1 inhibitor is advocated although controversially debated (Harrison & Barbui, 2011). A gain of function mutation of the JAK2 kinase (*JAK2*^{V617F}) has been identified in approximately half of the patients with PT, but a direct association between *JAK2*^{V617F} positivity, whole blood thrombotic potential and thrombotic events has not been clearly established (Dahabreh *et al*, 2009). A retrospective analysis claimed that aspirin therapy reduces the incidence of venous thromboses in *JAK2* positive patients (Alvarez-Larran *et al*, 2010). The purpose of our study was to determine whether aspirin reduces the thrombotic potential of patients to the levels achieved in healthy volunteers (HV) and whether the *JAK2*^{V617F} mutation is associated with whole blood thrombotic potential.

The whole blood thrombotic potential of a cohort of 12 PT patients, who were undergoing an antiplatelet therapy with aspirin (100 mg QD) was compared with that of 10 HV treated with the same regimen for 3 d prior to blood sampling. Whole blood thrombotic potential was tested in real time using a perfusion chamber assay, which allowed real time analysis of platelet deposition on collagen (Barstad *et al*, 1996). Whole blood was anti-coagulated with an activated Factor X (FXa) inhibitor to preserve physiological Ca^{++} concentration, and perfused over a fibrillar collagen (Type I; Chrono-Log Corporation, Havertown, PA, USA) surface at shear rates approximating those in veins (100/s), arteries (600/s) and moderately stenosed arteries (1600/s) (Betz *et al*, 1999). Platelets were labeled *in situ* using Rhodamine 6G (VWR International, Radnor, PA, USA) and the deposition of fluorescently labelled platelets on the collagen surface was monitored in real time (1 Hz) by fluorescence microscopy. Endpoint thrombosis (size of thrombi at $t = 300$ s expressed as Mean Fluorescent Intensity/Area of coverage) and rate of thrombus growth were calculated from the microscopy images (Gurbel *et al*, 2010) and recorded for each individual as measures of whole blood thrombotic potential. Data are expressed as Mean ± standard error of the mean (statistics performed with GRAPH PAD PRISM v4.03 [Graph Pad Software, La Jolla, CA, USA] using unpaired, two-tailed Students *t*-test). *P*-values <0·05 were considered to be statistically significant.

Three important observations were made: First, whole blood from PT patients formed larger thrombi than blood from HV despite daily aspirin treatment (Fig 1A–C). Second, increased platelet count in PT could be related to some, but not all of the differences observed between PT and HV at the different levels of shear (Fig 1D, E). Third, *JAK2* status does not influence the whole blood thrombotic potential (Fig 1F).

Whole blood from PT patients formed larger thrombi than those formed by HV despite aspirin treatment at all shear rates tested (Fig 2). Platelet turnover is increased in PT and circulating newly synthesized, reticulated platelets are less sensitive to aspirin as a QD aspirin dosing regime does not prevent formation of residual thromboxane A_2 (TXA_2). This