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The marrow stem cell niche in normal and malignant hematopoiesis

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Abstract

The hematopoietic niche is composed of endothelial cells, mesenchymal stromal cells of several types, and megakaryocytes, and functions to support the survival, proliferation, and differentiation of normal hematopoietic stem cells (HSCs). An abundance of evidence from a range of hematological malignancies supports the concept that the niche also participates in the pathogenesis of malignant hematopoiesis, differentially supporting malignant stem or progenitor cells over that of normal blood cell development. In 2005, patients with myeloproliferative neoplasms were reported to harbor an acquired, activating, missense V617F mutation of the cytokine-signaling Janus kinase (JAK)-2, JAK2^{V617F}, present in virtually all patients with polycythemia vera and half of patients with essential thrombocythemia and primary myelofibrosis. Using both *in vitro* and *in vivo* methods, several investigators have shown that in addition to driving cytokine-independent proliferation in HSCs, JAK2^{V617F} contributes to these neoplasms by altering the hematopoietic niche. The role of both endothelial cells and megakaryocytes bearing JAK2^{V617F} will be presented, which involves altering cytokine production within the niche, resulting in their differential support of mutant kinase-bearing stem cells over their normal counterparts, and imparting relative radiation resistance to stem cells. The clinical correlates of these findings will be discussed, as will their therapeutic implications.

Keywords

hematopoiesis; myeloproliferative neoplasms; Jak2V₆₁₇F; megakaryocytes; endothelial cells; hematopoietic stem cells; hematopoietic niche

Introduction

Accumulating evidence indicates that the survival and proliferation of hematological neoplasms are dependent not only on the cell-intrinsic functional properties of the driver mutations present within the cancer stem cell, but also on the ability of the surrounding cells to nurture the malignancy. Recent studies from a host of hematological malignancies have established the principle that the hematopoietic stem cell (HSC) niche can promote the expansion of neoplastic cells and suppress normal blood cell production, and these

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Competing interests

The authors declare no competing interests.

promalignancy properties are imparted to the niche by leukemic stem cells (LSCs) or their progeny. Other *in vitro* studies and the results of conditioning therapy for stem cell transplantation of patients with myeloproliferative neoplasms (MPNs) suggest that neoplastic hematopoiesis is radio- and chemotherapy-resistant, and that the niche may be at least partially responsible for this phenotype as well. It is hoped that a better understanding of the molecular and cellular events responsible for these features of these malignancies will lead to better treatment for patients with MPNs.

In order to explore such properties in MPNs, several investigators have generated murine models of these disorders using the *JAK2^{V617F}* oncogene, encoding a kinase mutant present in nearly every patient with polycythemia vera (PV), and about half of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF). We used a *JAK2^{V617F}* transgene that can only activate if the cell also expresses a Cre recombinase, allowing us to express the oncogene in specific cell types. In this way, we could assess the contribution to neoplastic stem cell growth of oncogene-bearing endothelial cells (ECs) and megakaryocytes (MKs).

The *JAK2^{V617F}* vascular niche promotes *JAK2^{V617F}*-mutant clonal expansion over normal HSCs, both *in vitro* and *in vivo*

To study the effects of the *JAK2^{V617F}* mutation on perivascular niche function, we crossed mice that bear a Cre-inducible human *JAK2^{V617F}* transgene Flip-Flop (FF1)¹ with *Tie2-Cre* transgenic mice² to express *JAK2^{V617F}* specifically in all hematopoietic cells (including HSCs) and ECs (*Tie2⁺/FF1⁺*).^{3,4} It is clear that both mature blood cells and HSCs in patients with MPNs express *JAK2^{V617F}*. However, recent work has indicated that ECs in patients with PV, ET, and PMF also express the kinase mutant.^{5–7} Moreover, as we and others have found that MKs play an important niche function in normal hematopoiesis,^{4,8,9} by using several murine transgenic strains of Cre recombinases and reciprocal marrow transplantation studies, we could explore the role of mutant ECs and of mutant MKs on niche function in the development of MPNs both *in vitro* and *in vivo*.

First, to assess the growth of normal and malignant hematopoietic cells on normal or malignant ECs, we cocultured normal and/or *JAK2^{V617F}*-positive, lineage negative, c-Kit positive (*Lin⁻/cKit⁺*) marrow cells (MCs) on a monolayer of normal or *JAK2^{V617F}*-positive ECs. We found no difference between normal or *JAK2^{V617F}*-mutated *Lin⁻/cKit⁺* cell proliferation when cocultured on normal ECs. In contrast, *JAK2^{V617F}* *Lin⁻/cKit⁺* cells displayed a relative growth advantage over the corresponding normal *Lin⁻/cKit⁺* cells when cocultured on *JAK2^{V617F}*-positive ECs.¹⁰ These results suggested that the malignant niche differentially supports the proliferation of malignant stem cells over normal cells.

Next, in order to assess whether the enhanced proliferation of *JAK2^{V617F}* *Lin⁻/cKit⁺* cells on *JAK2^{V617F}* ECs is also seen *in vivo*, we performed competitive marrow transplantation experiments in which donor MCs from *Tie2⁺/FF1⁺* mice were injected intravenously together with competitor normal MCs (using *CD45.1/45.2* alleles to track cell origins) into lethally irradiated *Tie2⁺/FF1⁺* mice or irradiated control recipients. During a 4-month follow-up, recipients of *Tie2⁺/FF1⁺* MCs displayed a greater level of peripheral blood *JAK2^{V617F}* expansion than when transplanted into control recipients. By 18 weeks post-transplant, *Tie2⁺/FF1⁺* recipients of an equal mixture of *Tie2⁺/FF1⁺* and wild-type cells

developed a profound MPN phenotype with neutrophilia, thrombocytosis, and moderate splenomegaly.¹¹ Quantitative evaluation of the marrow HSC compartment at that time revealed significant increases in JAK2^{V617F} CD150⁺CD48⁻ cells, a population of cells highly enriched in HSCs (~20% display long-term repopulating capacity), in Tie2⁺/FF1⁺ recipients compared with control recipients. In contrast, and somewhat surprisingly, control recipients (with the normal stem cell niche) of an equal mixture of Tie2⁺/FF1⁺ and normal cells had mostly normal blood cell counts, and there were no significant differences between the numbers of normal and mutant CD150⁺CD48⁻ cells in the marrow of the mice. Therefore, the JAK2^{V617F} vascular niche ECs promoted the expansion of JAK2^{V617F} HSCs at the expense of normal hematopoiesis. In contrast to these results, Wernig and colleagues reported that the transplantation of retrovirally expressed JAK2^{V617F} in MCs, which were then transplanted into normal mice, resulted in a lethal polycythemia/myelofibrosis phenotype.¹² The difference between these and our results could be the (almost certain) far higher level of JAK2^{V617F} expressed from the retrovirus versus that found in our transgene experiments, or the presence of normal HSCs in our animal model, where a 1:1 mix of normal and JAK2^{V617F}-positive MCs were transplanted.

The JAK2^{V617F}-positive ECs protect JAK2^{V617F} HSCs from radiation injury both *in vitro* and *in vivo*

Relapse following myeloablative transplantation of patients with MPNs is common, seen more frequently than in patients with *de novo* acute myelogenous or lymphocytic leukemia in first remission, or several other hematological malignancies. To investigate the effects of the JAK2^{V617F} vascular niche on the response of MPN HSCs to radiation often used in clinical transplantation, we transplanted normal MCs directly into lethally irradiated (950 cGy, a dose that in our hands virtually always results in full donor engraftment) Tie2⁺/FF1⁺ mice or age-matched normal recipients. During a 3-month follow-up, while all normal recipients displayed full donor engraftment, 7 of 12 (~60%) Tie2⁺/FF1⁺ recipient mice displayed recovery of JAK2^{V617F}-mutant hematopoiesis (mixed donor/recipient chimerism) 10 weeks after irradiation and transplantation of normal MCs. In contrast to the five Tie2⁺/FF1⁺ recipients with full donor engraftment, the seven mice with mixed chimerism developed neutrophilia, thrombocytosis, splenomegaly, and mutant HSC expansion, similar to what we observed in the primary Tie2⁺/FF1⁺ JAK2^{V617F}-mutant mice.^{3,11} Thus, JAK2^{V617F} HSCs in Tie2⁺/FF1⁺ mice were relatively protected from an otherwise lethal dose of irradiation administered during preparation for marrow transplantation. In essence, the presence of JAK2^{V617F} generated a model of disease refractory to what should have been curative transplantation.

As noted above, *Tie2-Cre* mice express Cre recombinase in both ECs and all HSCs and their progeny. To investigate whether the radioprotection phenotype we observed was due to the presence of JAK2^{V617F} in Tie2⁺/FF1⁺ HSCs, we generated a chimeric murine model with JAK2^{V617F}-mutant HSCs and the wild-type vascular niche, by transplanting Tie2⁺/FF1⁺ MCs into normal recipients and testing for radiation sensitivity. Transplantation of normal MCs into normal, irradiated recipients served as a control. Following hematopoietic recovery and full donor cell engraftment, each set of mice was again irradiated with 300 cGy, to create radiation injury, and 2 h later, marrow Lin⁻ cells were isolated for evaluation

of cellular apoptosis. We found that, in the presence of the wild-type vascular niche, cell apoptosis was significantly higher in the JAK2^{V617F}-mutant cells compared with normal cells, not decreased. Thus, radioresistance in mutant HSCs alone did not appear to be responsible for the mixed chimerism seen (equivalent to a relapse) following marrow transplantation. To further investigate the effects of the JAK2^{V617F} vascular niche on stem and progenitor cell response to radiation injury *in vivo*, we performed the converse experiment, generating chimeric mice with normal HSCs and JAK2^{V617F} ECs, by transplanting normal MCs into lethally irradiated (950 cGy) Tie2⁺/FF1⁺ recipients. The transplantation of normal MCs into normal recipients again served as a control experiment. Six to 10 weeks following transplantation, each set of mice was again irradiated with 300 cGy to create radiation injury. Two hours later, marrow Lin⁻ cells were isolated for evaluation of cellular apoptosis. We found that normal Lin⁻ cell apoptosis was decreased in mice with JAK2^{V617F}-positive compared with normal ECs,¹³ and the protection against apoptosis was even more striking when JAK2^{V617F}-mutant MCs were irradiated in the presence of JAK2^{V617F}-mutant ECs, which resulted in a ~ 50% reduction.

We then tested whether ECs bearing the JAK2^{V617F} mutation promoted HSC radioprotection more directly. Lin⁻ MCs were isolated from wild-type or Tie2⁺/FF1⁺ mice and cultured on primary EC feeder layers derived from wild-type or Tie2⁺/FF1⁺ murine lungs. The cocultures were then irradiated with 300 cGy and the hematopoietic cells were counted and assessed for colony forming capacity 24 h after irradiation. We observed higher total hematopoietic cell numbers and colonyforming progenitors from JAK2^{V617F} MCs cultured on JAK2^{V617F} ECs compared with their being cultured on normal ECs, suggesting that the JAK2^{V617F} vascular niche contributed directly to the JAK2^{V617F}-mutant MC radioprotection. Taken together, these *in vitro* and *in vivo* data indicate that the JAK2^{V617F} vascular niche contributes directly to HSC radioprotection, which could be responsible for the high incidence of disease relapse in patients undergoing allogeneic stem cell transplantation for MPNs.^{14–16}

The JAK2^{V617F} mutation expands the perivascular niche

Next, we attempted to determine the cellular basis of how the JAK2^{V617F} mutation alters ECs to promote neoplastic hematopoiesis in MPNs. First, we found that the JAK2^{V617F} ECs (isolated from the lungs of Tie2⁺/FF1⁺ mice) displayed significantly increased cell proliferation, wound closure (as a measure of cell migration), tube formation (as a measure of angiogenesis), and decreased apoptosis (after irradiation) compared with normal ECs *in vitro*,¹⁰ indicating that the kinase mutant might act to expand the vascular niche in JAK2^{V617F}-positive Tie2⁺/FF1⁺ mice, and alters its function, findings that also characterize the marrow of patients with MPNs.^{17–19}

Based on the known chemokine- and cytokine-mediated mechanisms by which C-X-C motif chemokine ligand 12 (CXCL12)-abundant reticular (CAR) niche cells support hematopoiesis, we then measured the expression levels of CXCL12 and stem cell factor (SCF), the two essential niche factors important for HSC maintenance and survival,^{20–23} in freshly isolated marrow ECs from normal and Tie2⁺/FF1⁺ mice. Both quantitative polymerase chain reaction (qPCR) analysis and flow cytometry revealed that compared with

normal ECs, both CXCL12 and SCF were upregulated in JAK2^{V617F}-positive marrow ECs. Moreover, the proportion of CD150⁺CD48⁻ cells expressing the CXCL12 receptor CXCR4, or the SCF receptor, c-Kit, was significantly increased in the marrow of JAK2^{V617F} compared with control mice. Therefore, increased CXCL12 and SCF levels in the JAK2^{V617F}-positive ECs could contribute to the clonal expansion of JAK2^{V617F}-positive HSCs, via the upregulated CXCR4 and c-Kit receptors, over that seen in normal animals. To determine if enhanced cytokine and/or cytokine receptors might be, at least in part, responsible for the relative radioresistance seen in Tie2⁺/FF1⁺ mice, we found that the expression levels of CXCL12, epidermal growth factor,²⁴ and pleiotrophin²⁵ were also upregulated in irradiated JAK2^{V617F} ECs compared with that seen in normal ECs.¹³ These results suggest that the JAK2^{V617F} vascular niche contributes to JAK2^{V617F} HSC expansion and radioprotection by its expression of HSC-active chemokines and cytokines. Of some note, the cytokine profile in a murine model of chronic myelogenous leukemia was found altered and was responsible for the relative growth advantage of the LSCs over that of their normal counterparts.²⁶

Thrombopoietin and its receptor c-Mpl are important for the malignancy-enhancing effects of the JAK2^{V617F} perivascular niche

Previously, work from our group and others has demonstrated that thrombopoietin (THPO) and the THPO receptor c-Mpl are essential for neoplastic HSC expansion and the development of an MPN in Tie2⁺/FF1⁺ mice,²⁷ and Ng and colleagues found that driving THPO levels up by deletion of *c-Mpl* in mature cells was essential for the development of an MPN in mice.²⁸ In another work, we and others established that MKs are a critical component of the perivascular niche.^{27,29–31} For example, we crossed FF1 mice with platelet factor 4 (PF4)-Cre mice,³² where the recombinase is expressed exclusively in MKs and platelets, in order to restrict JAK2^{V617F} expression exclusively to the MK lineage. As expected, the PF4⁺/FF1⁺ mice developed modest thrombocytosis, splenomegaly, and greatly increased marrow megakaryopoiesis, but surprisingly, these mice also developed a significant increase in CD45⁺EPCR⁺CD48⁻CD150⁺ (E-SLAM) cell numbers,⁴ a highly purified long-term repopulating HSC population.^{33,34} A highly sensitive RT-PCR assay was constructed to be certain that the PF4 promoter was not leaky, driving the expression of JAK2^{V617F} in HSCs; the assay conclusively demonstrated that JAK2^{V617F} was never detected in cells other than MKs or platelets. To determine whether THPO and c-Mpl were essential for this phenotype, or that of the Tie2⁺/FF1⁺ mice, we then ablated THPO or c-Mpl in PF4⁺/FF1⁺ by crossing the PF4⁺/FF1⁺ mice with *Thpo*^{-/-} mice³⁵ or c-Mpl^{-/-} mice³⁶ and found that neither the THPO⁻/PF4⁺/FF1⁺ nor the c-Mpl⁻/PF4⁺/FF1⁺ mice developed significant thrombocytosis or expanded their HSC compartment during more than 1 year of follow-up. Yoshihara and colleagues also found that c-Mpl was critical for the interaction of stem cells with the marrow niche.³⁷ Thus, neither MK nor E-SLAM expansion was observed in either strain of mice, despite the presence of the JAK2^{V617F}-positive MKs in both.³⁸ Taken together, these studies demonstrated that THPO/c-Mpl signaling is important for MPN HSC expansion in both Tie2⁺/FF1⁺ and PF4⁺/FF1⁺ mice, where HSCs and/or major components of the hematopoietic perivascular niche (ECs and MKs in Tie2⁺/FF1⁺, and MKs in PF4⁺/FF1⁺, respectively) bear the JAK2^{V617F} mutation.

THPO and c-Mpl are important for vascular endothelial cell function

c-Mpl is expressed on several types of ECs.^{39–41} Whether the EC c-Mpl receptor affects perivascular niche function, and contributes to the critical role of THPO/c-Mpl signaling in HSC maintenance, is not known. We measured EC c-Mpl expression by qPCR and found that compared with normal cells the receptor was upregulated in JAK2^{V617F}-positive lung and marrow ECs,^{10,11} suggesting that THPO/c-Mpl signaling could affect both the general vasculature (e.g., the lung) and the hematopoietic vascular niche (e.g., the marrow) in JAK2^{V617F}-positive MPNs. We also examined the effect of THPO on EC function *in vitro* and found that the cytokine significantly stimulated EC cell migration in a dose-dependent fashion. In addition, compared with untreated ECs, both the EC junction molecules zonula occludens-1⁴² and platelet EC adhesion molecule 1 (orCD31)⁴³ were upregulated in THPO-treated ECs, again suggesting that c-Mpl signaling may regulate vascular integrity, which is important for both hemostasis and tissue stem cell function.^{42,44}

To test the hypothesis that EC c-Mpl is important for the JAK2^{V617F}-mutant cell expansion in the perivascular niche, we designed an *in vitro* competitive growth assay where both normal and JAK2^{V617F} Lin[−]/cKit⁺ cells were cultured together (1:1 mix) in the presence of EC-conditioned medium (ECCM) collected from either normal or *c-Mpl*^{−/−} murine lung ECs. At the end of the 6-day culture, there were significantly more normal cells than JAK2^{V617F} cells in the presence of *c-Mpl*^{−/−} ECCM compared with that seen when cultured with wild-type ECCM, suggesting that EC c-Mpl is important for the maintenance/expansion of the JAK2^{V617F} cells over normal cells *in vitro*.¹⁰ This observation led us to hypothesize that certain secreted factors from the vascular ECs contribute to the JAK2^{V617F} clonal expansion in MPNs and that c-Mpl signaling is critical for this perivascular niche function. Indeed, we found that CXCL12 expression was significantly downregulated in *c-Mpl*^{−/−} or *Thpo*^{−/−} marrow ECs compared with marrow ECs from normal mice. Since CXCL12 is important in directing MK migration toward the perivascular niche and promoting MK maturation and platelet release,^{45,46} the decreased expression of CXCL12 in *Thpo*^{−/−} and *c-Mpl*^{−/−} marrow ECs could impair the interactions between MKs and ECs in the vascular niche. Indeed, histological examination of the marrow revealed that MKs are less likely to be in direct contact with the sinusoidal vessels in the *Thpo*^{−/−} and *c-Mpl*^{−/−} mice compared with normal mice, even when controlled for MK number, suggesting that c-Mpl signaling can affect MK-EC interactions in the perivascular niche. These results indicate that c-Mpl signaling affects endothelial function in both the general vasculature and the hematopoietic perivascular niche.

Future directions: direct evaluation of the JAK2^{V617F} vascular niche using confocal immunofluorescence imaging and magnetic resonance imaging

To gain a better appreciation of the interactions between ECs and MKs, and vascular development in the MPNs more generally, we have developed two approaches. Work with Sean Morrison at the University of Texas Southwestern School of Medicine has begun to examine the JAK2^{V617F} perivascular niche using immunofluorescence staining and deep confocal imaging. Our preliminary studies show that the marrow MK-vascular contact is significantly increased in the Tie2⁺/FF1⁺ mice compared with wild-type control mice. Considering that most HSCs reside close to the marrow sinusoid and that MKs are often

located adjacent to sinusoids, altered interactions between MKs and ECs in the JAK2^{V617F} perivascular niche could play an important role in the neoplastic hematopoiesis of patients with MPNs. Moreover, since CXCL12 is important in directing MK migration toward the vascular niche, the increased CXCL12 levels in JAK2^{V617F} ECs could be responsible for this increased MK-EC contact in Tie2⁺/FF1⁺ mice, an active hypothesis that is being explored. This finding is quite consistent with the decrease in CXCL12 levels and in MK-EC interactions seen in *Thpo*^{-/-} and *c-Mpl*^{-/-} mice. In other work, through collaboration with Tim Duong at the Renaissance School of Medicine at Stony Brook University, we have employed advanced magnetic resonance imaging (MRI) methods to study both the structure and function of the JAK2^{V617F} marrow vasculature *in vivo*. MRI can provide a noninvasive serial assessment of marrow vascularity to supplement “snapshot” histological findings.⁴⁷ Our preliminary studies suggest that compared with control animals there is increased blood flow and vascular permeability in the JAK2^{V617F} perivascular niche of Tie2⁺/FF1⁺ mice. Armed with these two advanced imaging techniques, we are encouraged that high-resolution MRI and immunofluorescence staining and confocal imaging will provide an unprecedented approach to study the perivascular niche in normal and neoplastic hematopoiesis.

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