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# JAK2V617F-bearing vascular niche enhances malignant hematopoietic regeneration following radiation injury

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## ABSTRACT

Myeloproliferative neoplasms are clonal stem cell disorders characterized by hematopoietic stem/progenitor cell expansion. The acquired kinase mutation *JAK2V617F* plays a central role in these disorders. Abnormalities of the marrow microenvironment are beginning to be recognized as an important factor in the development of myeloproliferative neoplasms. Endothelial cells are an essential component of the hematopoietic vascular niche. Endothelial cells carrying the *JAK2V617F* mutation can be detected in patients with myeloproliferative neoplasms, suggesting that the mutant vascular niche is involved in the pathogenesis of these disorders. Here, using a transgenic mouse expressing *JAK2V617F* specifically in all hematopoietic cells (including hematopoietic stem/progenitor cells) and endothelial cells, we show that the *JAK2V617F*-mutant hematopoietic stem/progenitor cells are relatively protected by the *JAK2V617F*-bearing vascular niche from an otherwise lethal dose of irradiation during conditioning for stem cell transplantation. Gene expression analysis revealed that chemokine (C-X-C motif) ligand 12, epidermal growth factor, and pleiotrophin are up-regulated in irradiated *JAK2V617F*-bearing endothelial cells compared to wild-type cells. Our findings suggest that the mutant vascular niche may contribute to the high incidence of disease relapse in patients with myeloproliferative neoplasms following allogeneic stem cell transplantation, the only curative treatment for these disorders.

## Introduction

The chronic Philadelphia chromosome (Ph1) negative myeloproliferative neoplasms (MPNs) are clonal stem cell disorders characterized by hematopoietic stem/progenitor cell (HSPC) expansion and overproduction of mature blood cells. The acquired kinase mutation *JAK2V617F* plays a central role in MPNs. However, the mechanisms responsible for the malignant HSPC expansion in MPNs are not fully understood, limiting the effectiveness of current treatment. Although the etiology of dysregulated hematopoiesis has been mainly attributed to the molecular alterations within the HSPC compartment, abnormalities of the marrow microenvironment are beginning to be recognized as an important factor in the development of MPNs.<sup>1-5</sup> The diseased niche could impair normal hematopoiesis and favor the competing malignant stem cells, which could contribute to the poor donor engraftment and high incidence of disease relapse following allogeneic stem cell transplantation (SCT), the only curative treatment for patients with MPNs.<sup>2,6-8</sup>

Endothelial cells (ECs) are an essential component of the hematopoietic niche and most HSPCs reside close to a marrow sinusoid (the “perivascular niche”).<sup>9</sup> MPNs are characterized by increased marrow angiogenesis compared to normal marrow.<sup>10-12</sup> Although the existence and cell of origin of endothelial progenitors is still a subject of debate, *JAK2V617F* mutation can be detected in endothelial progenitors derived from the hematopoietic lineage (the so-called endothelial cell

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colony-forming units; CFU-ECs or Hill) and, in some reports, in the true endothelial colony-forming cells (ECFC) based on *in vitro* assays.<sup>13-17</sup> *JAK2V617F* mutation is also present in isolated liver or spleen ECs from patients with MPNs.<sup>15,18</sup> Previously, we and others have shown that *JAK2V617F*-bearing ECs are critical in the development of the bleeding abnormalities in a murine model of *JAK2V617F*-positive MPNs in which *JAK2V617F* is expressed in all hematopoietic cells and endothelial cells.<sup>19</sup> In addition, we have reported that the *JAK2V617F*-bearing vascular niche promotes the expansion of the *JAK2V617F* HSPCs in preference to *JAK2WT* HSPCs.<sup>20,21</sup> All of these observations suggest that ECs are involved in the pathogenesis of MPNs.

In the present study, using the hematopoietic and endothelial specific Tie2-Cre system and different marrow transplantation models, we demonstrate that *JAK2V617F*-mutant HSPCs are relatively protected by the *JAK2V617F*-bearing vascular niche from the otherwise lethal irradiation administered during conditioning for marrow transplantation. Taken together, our studies indicate that the mutant vascular niche could contribute to the poor donor cell engraftment and the high incidence of disease relapse well known to occur in patients with MPNs after allogeneic SCT. Therefore, targeting the altered hematopoietic vascular niche could provide more effective therapies for patients with MPNs.

## Methods

### Experimental mice

*JAK2V617F* Flip-Flop (FF1) mice<sup>22</sup> were provided by Radek Skoda (University Hospital, Basal, Switzerland) and Tie2-Cre mice<sup>23</sup> by Mark Ginsberg (University of California, San Diego, USA). The FF1 mice were crossed with Tie2-Cre mice to express *JAK2V617F* specifically in hematopoietic cells and ECs (Tie2/FF1 mice). All mice used were crossed onto a C57BL/6 background and were bred in a pathogen-free mouse facility at Stony Brook University. CD45.1<sup>+</sup> congenic mice (SJL) were purchased from Taconic Inc. (Albany, NY, USA). Animal experiments were performed in accordance with the guidelines provided by the Institutional Animal Care and Use Committee.

### Stem cell transplantation assays

The effects of the *JAK2V617F*-bearing vascular niche on MPN hematopoiesis were studied *in vivo* using marrow transplantation assays. First, we transplanted wild-type (WT) CD45.1 marrow cells into lethally irradiated (950cGy)<sup>24,25</sup> 8-14-week old Tie2/FF1 mice or WT controls (CD45.2). Peripheral blood was obtained every four weeks after transplantation, and CD45.1 donor chimerism and complete blood counts were measured.

To study the effects of HSPC *JAK2V617F* mutation on HSPC radioprotection, we generated a chimeric murine model with *JAK2V617F*-mutant HSPCs and WT vascular niche by transplanting *JAK2V617F* marrow cells (CD45.2) into lethally irradiated (950cGy) WT recipients (CD45.1). The transplantation of CD45.2 WT marrow cells into CD45.1 WT recipients served as a control. Following hematopoietic recovery and full donor cell engraftment, each set of mice were irradiated with 300cGy to create a radiation injury. Two hours later, marrow Lineageneg (Lin<sup>-</sup>) HSPCs were isolated using Lineage Cell Depletion Kit (Miltenyi Biotec, San Diego, CA, USA) for evaluation of cellular apoptosis and cell cycle status. For competitive marrow transplantation experiments, 5x10<sup>5</sup> post-irradiated marrow cells (CD45.2) were injected intra-

venously together with 1x10<sup>5</sup> competitor CD45.1 WT marrow cells into lethally irradiated (950 cGy) CD45.1 recipients. Peripheral blood was obtained every four weeks after transplantation, and CD45.2 chimerism was measured.

To study the effects of EC *JAK2V617F* mutation on HSPC radioprotection, we generated a chimeric murine model with WT HSPCs and *JAK2V617F*-bearing vascular niche by transplanting WT marrow cells (CD45.1) into lethally irradiated (950cGy) Tie2/FF1 recipients (CD45.2). The transplantation of CD45.1 WT marrow cells into CD45.2 WT recipients served as a control. Following hematopoietic recovery and full donor cell engraftment, each set of mice were irradiated with 300cGy to create a radiation injury. Two hours later, marrow Lineageneg (Lin<sup>-</sup>) HSPCs were isolated for evaluation of cellular apoptosis.

Additional details of the methods used can be found in the *Online Supplementary Methods*.

## Results

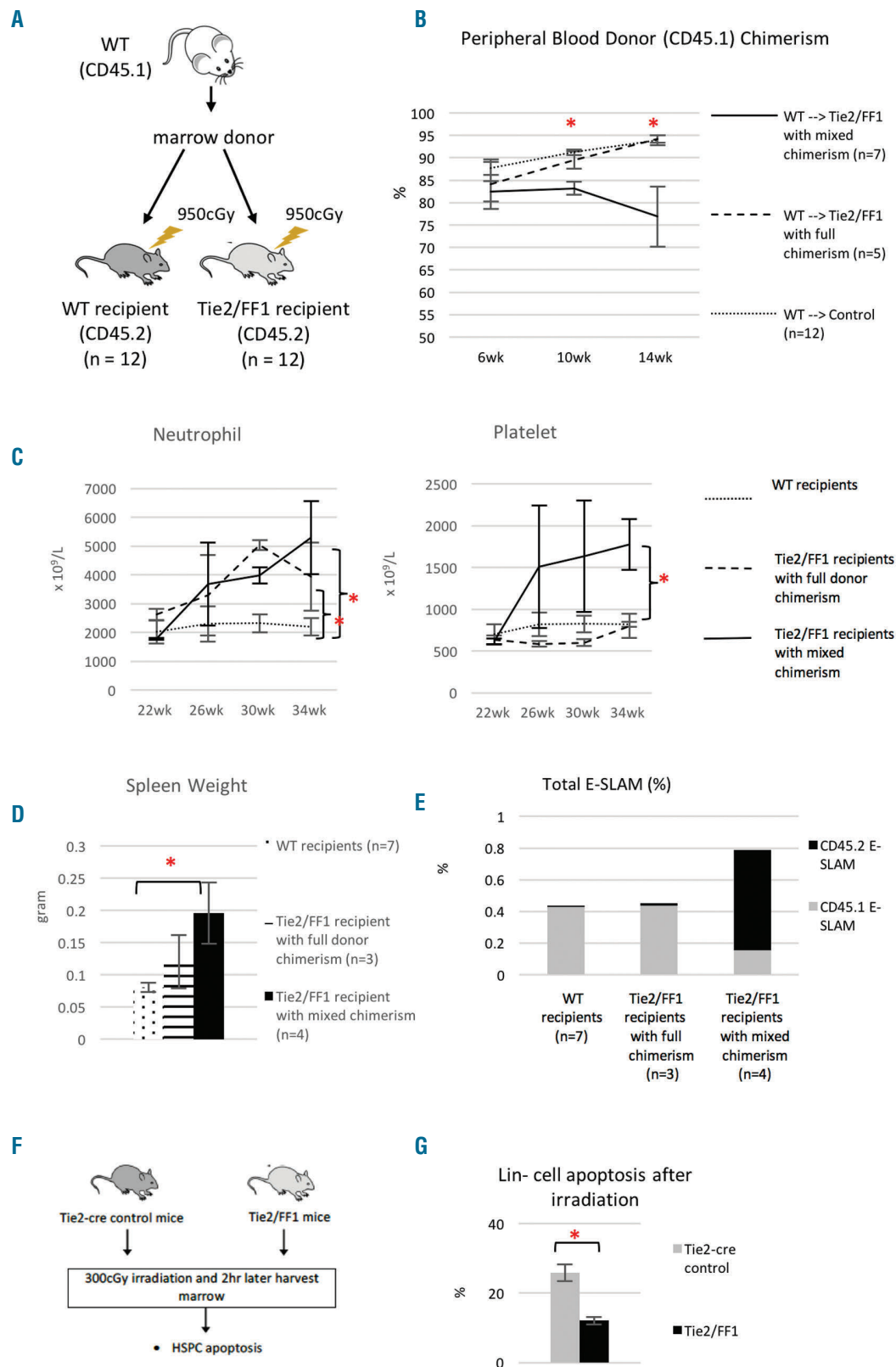
### Expression of *JAK2V617F* in Tie2<sup>+</sup> cells protects marrow HSPCs from lethal irradiation

Mice expressing Cre under the control of the Tie2 promoter (Tie2-Cre) were crossed with *JAK2V617F* Flip-Flop (FF1) mice to generate mice bearing human *JAK2V617F* expression specifically in endothelial and hematopoietic cells (Tie2/FF1). The Tie2/FF1 mice develop an MPN-like phenotype with neutrophilia, thrombocytosis, significant splenomegaly, and greatly increased marrow vascular density, megakaryopoiesis, and numbers of HSPCs.<sup>19,20</sup>

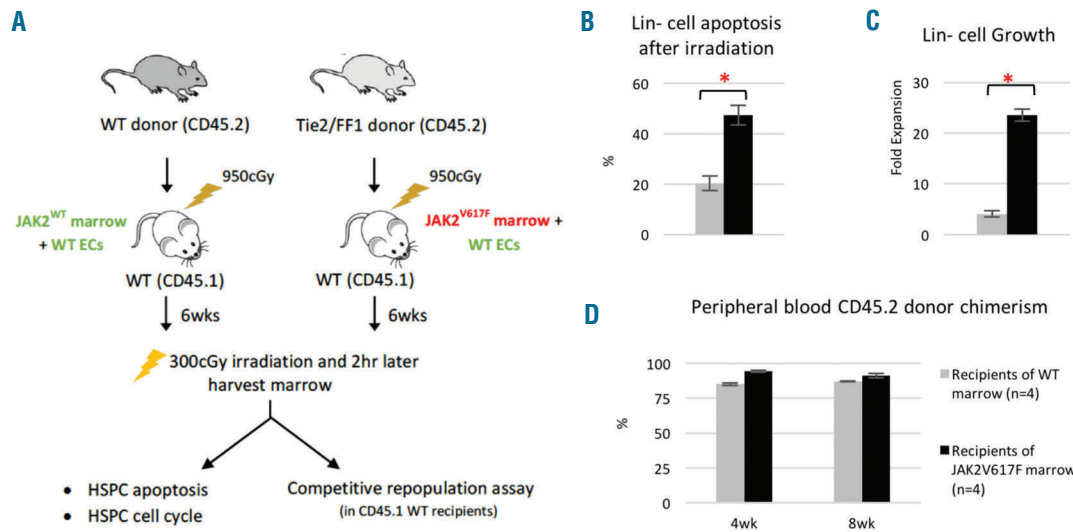
To investigate the effects of the *JAK2V617F*-bearing vascular niche on MPN hematopoiesis *in vivo*, WT CD45.1 marrow cells were transplanted directly into lethally irradiated (950cGy) Tie2/FF1 mice or age-matched littermate control mice (CD45.2) (n=12 in each group) (Figure 1A). During a 3-month follow up, while all WT control recipients displayed full donor engraftment, 7 of 12 (approx. 60%) Tie2/FF1 recipient mice displayed recovery of *JAK2V617F*-mutant hematopoiesis (mixed donor/recipient chimerism) ten weeks after transplantation (Figure 1B).

We followed some of the Tie2/FF1 and WT control recipients (n=7 in each group) for more than eight months. In contrast to the Tie2/FF1 recipients with full donor engraftment, the mixed chimeric mice developed neutrophilia, thrombocytosis, and splenomegaly (Figure 1C and D), similar to what has been observed in the primary Tie2/FF1 mice.<sup>19,26</sup> Flow cytometry analysis revealed that *JAK2V617F*-mutant CD45.2<sup>+</sup>EPCR<sup>+</sup>CD48<sup>+</sup>CD150<sup>+</sup> (E-SLAM) cells, which is a highly purified long-term repopulating HSPC population in normal and in MPN marrow,<sup>27,28</sup> are significantly expanded in the mixed chimeric mice compared to Tie2/FF1 recipients with full donor engraftment or WT recipients (Figure 1E).

In virtually all our transplantation experiments performed over the past four years, we have used 950cGy radiation<sup>24</sup> and have seen virtually 100% donor engraftment in every recipient. In contrast, 7 of 12 mice in our Tie2/FF1 recipients of normal marrow demonstrated mixed chimerism with an average of 23% recipient cells in peripheral blood at fourteen weeks following 950cGy irradiation and marrow transplantation, and developed an MPN phenotype resembling the primary Tie2/FF1 mice during more than eight months of follow up. These findings suggest that the *JAK2V617F*-mutant HSPCs in Tie2/FF1 mice are relatively protected from the otherwise



**Figure 1. *JAK2V617F* hematopoietic stem/progenitor cells (HSPCs) in the Tie2/FF1 mice are protected from lethal irradiation.** (A) Scheme of direct marrow transplantation. (B) Peripheral blood CD45.1 chimerism following transplantation of wild-type (WT) CD45.1 marrow cells into lethally irradiated Tie2/FF1 mice or WT control mice (CD45.2) ( $n=12$  in each group). (C) During more than eight months of follow up, Tie2/FF1 recipients with mixed chimerism developed both neutrophilia and thrombocytosis. (D) Spleens collected eight months following transplantation did not display significant weight differences between Tie2/FF1 recipients with full donor chimerism and WT recipients. In contrast, Tie2/FF1 recipients with mixed chimerism developed moderate splenomegaly compared to WT recipients (spleen weight 196 mg vs. 80 mg;  $P=0.010$ ). (E) The frequency of normal marrow donor-derived E-SLAM (CD45.1) cells in the Tie2/FF1 recipients was unchanged from WT recipient mice. In contrast, *JAK2V617F*-mutant recipient-derived E-SLAM (CD45.2) cells were significantly expanded in Tie2/FF1 recipients with mixed chimerism. (F) Schematic diagram of irradiation and analysis of Tie2/FF1 and WT control mice. (G) Lin<sup>-</sup> HSPC cell apoptosis in Tie2-cre control or Tie2/FF1 mice after 300cGy irradiation ( $n=2$  in each group).  $*P<0.05$ .



**Figure 2. The *JAK2V617F*-mutant hematopoietic stem/progenitor cell (HSPC) is more sensitive to radiation-induced apoptosis than *JAK2WT* HSPC.** (A) Outline of experiment design to generate a chimeric murine model with *JAK2V617F*-mutant HSPCs and wild-type (WT) vascular niche. (B) After 300cGy irradiation, cell apoptosis (Annexin V<sup>+</sup>7AAD<sup>+</sup>) was significantly increased in *JAK2V617F* Lin<sup>-</sup> HSPCs (black bar) compared to *JAK2WT* Lin<sup>-</sup> HSPCs (gray bar). (C) Unirradiated *JAK2V617F* (black) Lin<sup>-</sup> HSPCs proliferated to a greater extent than *JAK2WT* (gray) Lin<sup>-</sup> HSPCs *in vitro*. (D) Peripheral blood donor chimerism following a competitive repopulation assay in which 5x10<sup>5</sup> post-irradiated *JAK2WT* or *JAK2V617F* marrow cells (CD45.2) were injected together with 1x10<sup>5</sup> competitor CD45.1 WT marrow cells into lethally irradiated CD45.1 WT recipients (n=4 in each group). \*P<0.05.

lethal irradiation administered during conditioning for marrow transplantation. To confirm this hypothesis, we irradiated primary Tie2/FF1 mice or Tie2-cre control mice with 300cGy, and two hours later their marrow Lin<sup>-</sup>HSPCs were isolated for evaluation of cellular apoptosis (Figure 1F). We found that the *JAK2V617F* HSPCs in the mutant vascular niche (i.e. Tie2/FF1 mice) had significantly less cellular apoptosis compared to *JAK2V617F* HSPCs in WT vascular niche (i.e. control mice) (12.1% vs. 25.8%; P=0.043) (Figure 1G). Therefore, the *JAK2V617F*-mutant HSPCs in Tie2/FF1 mice are relatively protected from lethal irradiation, which could be responsible for the reported high incidence of disease relapse in patients undergoing allogeneic SCT for MPNs.<sup>2,6,7</sup>

**The *JAK2V617F*-mutant HSPC is more sensitive to radiation-induced apoptosis than *JAK2WT* HSPC**

Tie2-Cre mice express Cre recombinase in both ECs and hematopoietic cells. To investigate whether the radioprotection phenotype noted in the prior experiments is due to the *JAK2V617F* mutation in Tie2/FF1 HSPCs, we generated a chimeric murine model with *JAK2V617F*-mutant HSPCs and a WT vascular niche by transplanting Tie2/FF1 marrow cells into WT recipients. The transplantation of WT marrow cells into WT recipients served as a control. Following hematopoietic recovery and full donor cell engraftment, each set of mice were again irradiated with 300cGy to create a radiation injury, and two hours later, marrow Lineageneg (Lin<sup>-</sup>) HSPCs were isolated for evaluation of cellular apoptosis and cell cycle status. In our previous study, recipient mice of Tie2/FF1 marrow developed a MPN phenotype by eight weeks post transplantation with significant thrombocytosis and neutrophilia.<sup>19</sup> Therefore, we irradiated the mice at six weeks post transplant in this study before the development of any clinical phenotype (*data not shown*) (Figure 2A). We found that cell

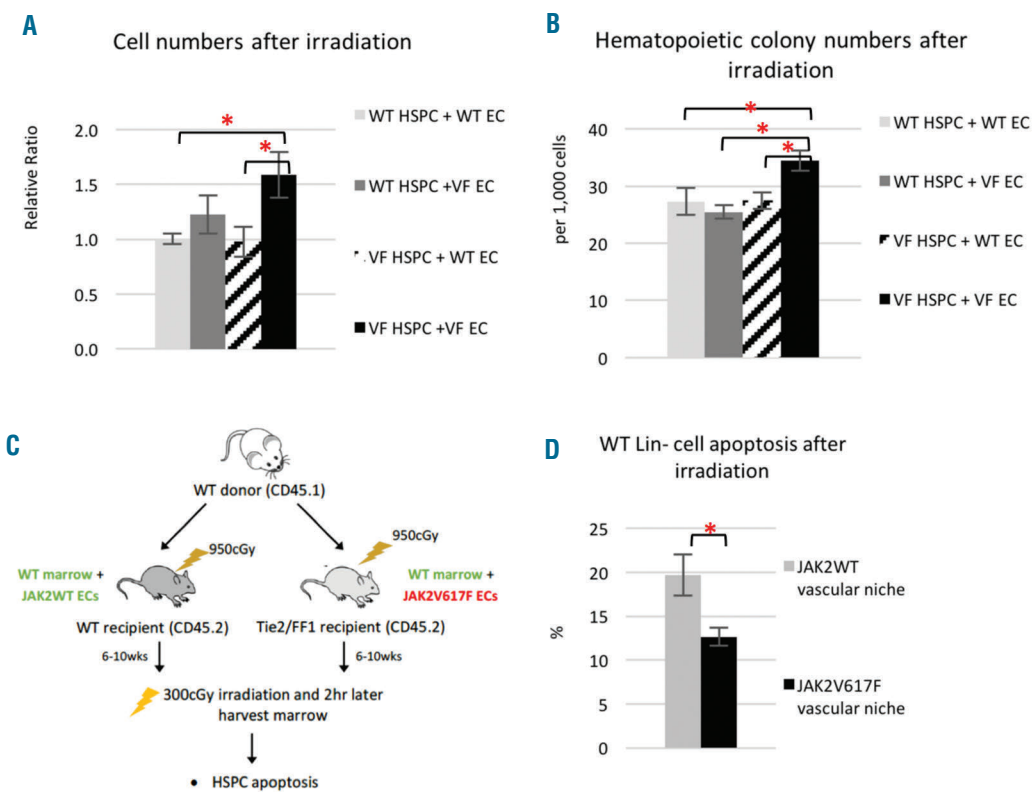
apoptosis was significantly increased in the *JAK2V617F*-mutant HSPCs compared to *JAK2WT* HSPCs (47.3% vs. 20.3%; P=0.005) (Figure 2B). This result is consistent with the observation that unirradiated *JAK2V617F* Lin<sup>-</sup> HSPCs proliferate to a greater extent than *JAK2WT* Lin<sup>-</sup> HSPCs in serum-free medium *in vitro* (5.8-fold; P=0.000006) (Figure 2C), and therefore are predictably more sensitive to radiation-induced apoptosis. There was no significant difference in cell cycle status between the *JAK2V617F* HSPCs and *JAK2WT* HSPCs after irradiation. These data suggest that, in the WT vascular niche, the *JAK2V617F*-mutant HSPC is more (not less) sensitive to radiation-induced apoptosis than are *JAK2WT* HSPCs.

To further test the effect of irradiation on HSPC function in the WT vascular niche, we performed a competitive repopulation assay in which 5x10<sup>5</sup> post-irradiated marrow cells (CD45.2 *JAK2WT* or CD45.2 *JAK2V617F*) were injected intravenously together with 1x10<sup>5</sup> competitor CD45.1 WT marrow cells into lethally irradiated (950 cGy) CD45.1 recipients (Figure 2A). Since the presence of *JAK2V617F* mutation in HSPCs may affect the cell's long-term proliferation, we focused on donor cell chimerism in the early phase of engraftment. During an 8-week post-transplant follow up, there was no difference in CD45.2 donor chimerism between the recipients of post-irradiated *JAK2V617F* marrow cells and recipients of post-irradiated *JAK2WT* marrow cells, suggesting that the engraftment potential of post-irradiated *JAK2V617F* HSPCs (or at least the short-term HSPCs) do not differ from *JAK2WT* HSPCs (Figure 2D).

***JAK2V617F*-bearing ECs protect HSPCs from lethal irradiation**

We next studied the effects of an EC *JAK2V617F* mutation on hematopoietic radioprotection. Lin<sup>-</sup> marrow HSPCs were isolated from WT or Tie2/FF1 mice and cul-





**Figure 3.** The *JAK2V617F*-bearing endothelial cells (ECs) protect *JAK2V617F* hematopoietic stem/progenitor cells (HSPCs) from lethal irradiation. (A and B) After 300cGy irradiation, there were higher total cell numbers (1.6-fold;  $P=0.026$ ) (A) and hematopoietic progenitors (1.3-fold;  $P=0.010$ ) (B) from *JAK2V617F* Lin<sup>-</sup> HSPCs cultured on *JAK2V617F*-bearing ECs compared to their being cultured on *JAK2WT* ECs. Cell numbers were shown as the relative ratio compared to *JAK2WT* Lin<sup>-</sup> HSPCs cultured on *JAK2WT* ECs under the same conditions. Data are from 3 independent experiments. (C) Outline of experiment design to generate a chimeric murine model with *JAK2WT* HSPCs and mutant vascular niche which is then subjected to sublethal irradiation. (D) CD45.1 WT Lin<sup>-</sup> HSPC cell apoptosis in WT or *JAK2V617F*-mutant vascular niche after 300cGy irradiation ( $n=4$  in each group).

tured on primary EC feeder layers derived from WT or Tie2/FF1 (*JAK2V617F*) murine lungs. The Lin<sup>-</sup> HSPC-EC co-cultures were irradiated with 300cGy *ex vivo* and cell number was counted within 24 hours of irradiation. We observed higher total cell numbers (1.6-fold;  $P=0.026$ ) and hematopoietic progenitors (1.3-fold;  $P=0.010$ ) from *JAK2V617F* HSPCs cultured on *JAK2V617F*-bearing ECs compared to their being cultured on *JAK2WT* ECs, suggesting that the *JAK2V617F*-bearing vascular niche contributes directly to *JAK2V617F*-mutant HSPC radioprotection (Figure 3A and B). No significant difference was observed in cell numbers or hematopoietic progenitors between *JAK2WT* HSPCs cultured on *JAK2V617F*-bearing ECs and their being cultured on *JAK2WT* ECs *in vitro* after irradiation.

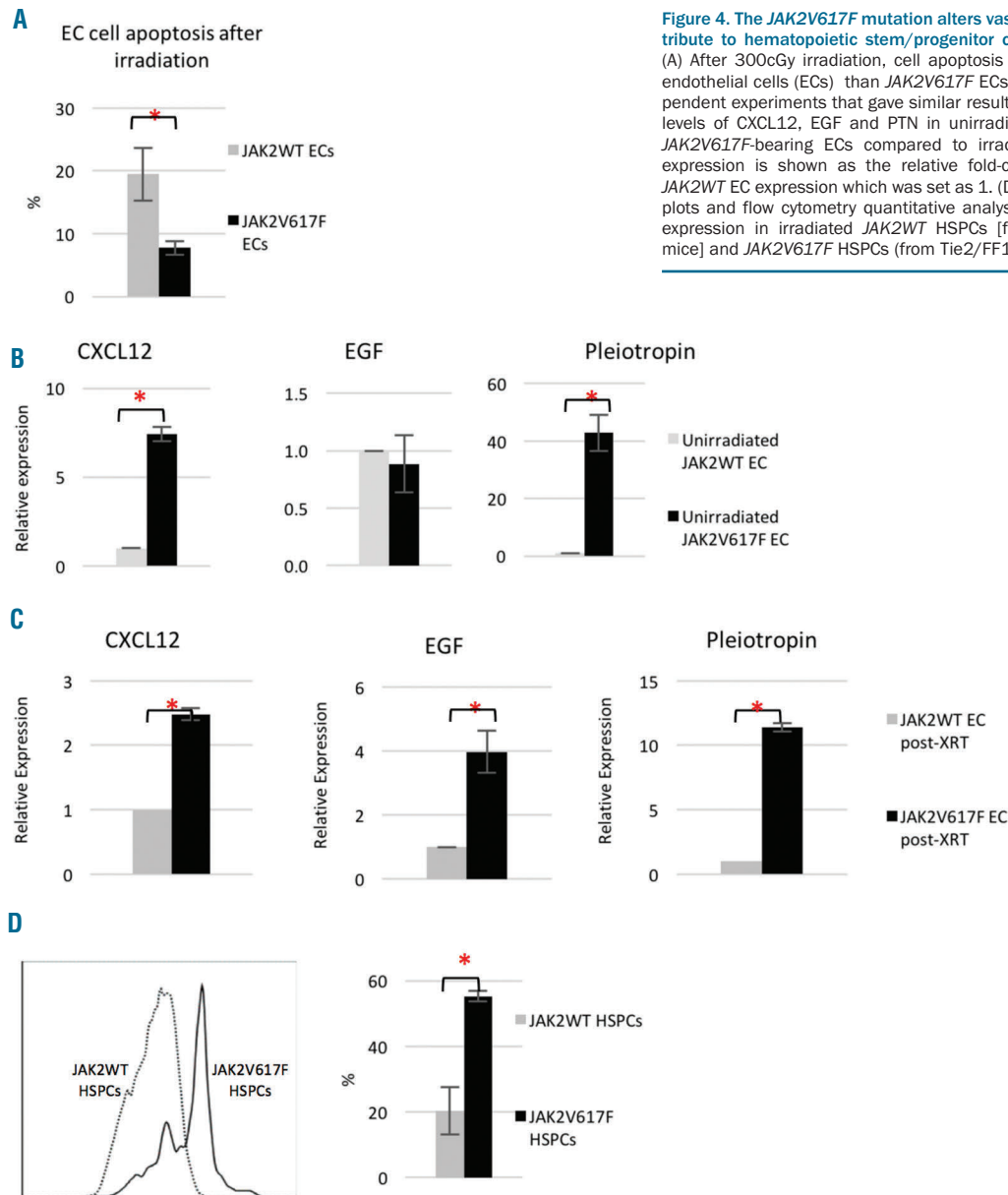
To further investigate the effects of *JAK2V617F*-bearing vascular niche on the response of HSPCs to radiation injury *in vivo*, we generated a chimeric murine model with WT HSPCs and *JAK2V617F*-bearing vascular niche by transplanting WT marrow cells (CD45.1) into lethally irradiated (950cGy) Tie2/FF1 recipients (CD45.2). The transplantation of CD45.1 WT marrow cells into CD45.2 WT recipients served as a control (Figure 3C). Based on our observation that Tie2/FF1 recipients did not develop any significant recovery of the *JAK2V617F*-mutant hematopoiesis until ten weeks after transplantation (Figure 1B), each set of chimeric mice were irradiated with 300cGy at 6-10 weeks following transplantation to create

a radiation injury. Two hours later, marrow Lin<sup>-</sup> HSPCs were isolated for evaluation of cellular apoptosis. We found that the WT Lin<sup>-</sup> HSPC cell apoptosis was decreased in the *JAK2V617F*-mutant vascular niche compared to WT vascular niche (12.7% vs. 19.7%;  $P=0.034$ ) (Figure 3D). Taken together, these data suggest that the *JAK2V617F*-bearing vascular niche contributes directly to HSPC radioprotection.

In contrast to our observations *in vivo*, where *JAK2V617F*-mutant HSPCs had increased apoptosis compared to *JAK2WT* HSPCs in the WT vascular niche (Figure 2A and B), cell numbers or progenitor numbers from the *JAK2V617F* HSPCs and *JAK2WT* HSPCs were similar when cultured on *JAK2WT* ECs (Figure 3A and B). Similarly, while the *JAK2V617F*-bearing vascular niche is protective for WT HSPCs *in vivo* (Figure 3C and D), *JAK2V617F* EC did not significantly protect WT HSPC *in vitro* (Figure 3A and B). These results are likely due to the different cell-cell interactions and niche factors between the *in vitro* culture condition and *in vivo* microenvironment.

#### The *JAK2V617F* mutation alters vascular niche function to contribute to HSPC radioprotection

Next, we investigated how the *JAK2V617F* mutation alters EC function in the vascular niche to protect HSPCs from radiation injury. In our previous studies, we found that *JAK2V617F*-bearing ECs proliferate to a greater extent than *JAK2WT* ECs and display significantly increased



**Figure 4. The *JAK2V617F* mutation alters vascular niche function to contribute to hematopoietic stem/progenitor cell (HSPC) radioprotection.** (A) After 300cGy irradiation, cell apoptosis was higher in the *JAK2WT* endothelial cells (ECs) than *JAK2V617F* ECs. Data are from 1 of 3 independent experiments that gave similar results. (B and C) The expression levels of CXCL12, EGF and PTN in unirradiated (B) and irradiated (C) *JAK2V617F*-bearing ECs compared to irradiated *JAK2WT* ECs. Gene expression is shown as the relative fold-change compared with the *JAK2WT* EC expression which was set as 1. (D) Representative histogram plots and flow cytometry quantitative analysis of phosphorylated EGFR expression in irradiated *JAK2WT* HSPCs [from wild-type (WT) control mice] and *JAK2V617F* HSPCs (from *Tie2/FF1* mice) (n=2 in each group).

angiogenesis *in vitro* compared to *JAK2WT* ECs. In addition, the tubular structures formed by the *JAK2V617F*-bearing ECs *in vitro* were more stable than those from *JAK2WT* ECs.<sup>21</sup> In this study, we found that *JAK2V617F* lung ECs displayed less cell apoptosis *in vitro* after 300cGy irradiation compared to *JAK2WT* ECs (7.7% vs. 19.5%;  $P=0.026$ ) (Figure 4A). It has long been known that hematopoietic recovery following lethal irradiation requires an intact vasculature.<sup>29-34</sup> Therefore, the increased cell proliferation and/or decreased apoptosis could expand the vascular niche in *JAK2V617F*-bearing mice, which in turn contributes to the hematopoietic radioprotection we have observed in the *Tie2/FF1* recipient mice.

CXCL12 is an essential niche factor important for both HSPC maintenance and HSPC survival after radiation injury.<sup>35-38</sup> Epidermal growth factor (EGF) and pleiotropin (PTN), two other factors secreted by the vascular niche,

have been shown to play important roles in the regulation of HSPC regeneration following radiation injury.<sup>39-41</sup> Recently, we demonstrated that the expression level of CXCL12 was up-regulated in *JAK2V617F*-bearing marrow ECs compared to wild-type ECs, which could mediate the clonal expansion of *JAK2V617F* HSPCs, via the up-regulated CXCL12 receptor CXCR4, over *JAK2WT* HSPCs.<sup>20</sup> To understand the EC signals responsible for HSPC radioprotection in the *Tie2/FF1* recipient mice, we measured the expression levels of CXCL12, EGF, and PTN in both non-irradiated and irradiated *JAK2WT* and *JAK2V617F* lung ECs. qPCR analysis confirmed that there was upregulation of CXCL12 (2.5-fold;  $P=0.0001$ ), EGF (4.0-fold;  $P=0.011$ ) and PTN (11.4-fold;  $P=0.00001$ ) in irradiated *JAK2V617F*-bearing ECs compared to irradiated *JAK2WT* ECs (Figure 4B and C). Furthermore, quantitative flow cytometry analysis showed that the proportion of marrow

CD150<sup>+</sup>CD48<sup>-</sup> HSPCs expressing phosphorylated EGFR was increased in irradiated *JAK2V617F* HSPCs (from Tie2/FF1 mice) as compared to irradiated *JAK2WT* HSPCs (from control mice) (2.7-fold;  $P=0.042$ ), suggesting that EGFR signaling activity was up-regulated in irradiated *JAK2V617F* HSPCs (Figure 4D). These results suggest that the *JAK2V617F*-bearing vascular niche contributes directly to HSPC radioprotection, possibly by its elaboration of soluble niche factors.

## Discussion

Vascular ECs are a major component of the HSPC niche (the “vascular niche”) and provide many key factors that are required for HSPC maintenance.<sup>9</sup> Patients with MPNs are characterized by increased marrow angiogenesis compared to normal marrow.<sup>10–12</sup> ECs carrying the *JAK2V617F* mutation can be detected in patients with MPNs, suggesting that ECs are involved in the pathogenesis of MPNs.<sup>15,18</sup> Here, by using the hematopoietic and endothelial specific Tie2-Cre system and different marrow transplantation models, we have been able to highlight the importance of *JAK2V617F*-bearing ECs in MPN disease relapse, which is seen in up to 40% of patients (especially after reduced intensity conditioning) following allogeneic SCT, the only curative treatment for MPNs.<sup>6,8,42–44</sup>

It has long been known that hematopoietic recovery following lethal irradiation requires an intact vasculature.<sup>29–34</sup> Following radiation injury, co-culture of irradiated HSPCs with ECs can rescue HSPCs with multilineage reconstituting capacity.<sup>45,46</sup> Our previous study has demonstrated that *JAK2V617F*-bearing ECs proliferate to a greater extent than *JAK2WT* ECs *in vitro*.<sup>21</sup> In this study, we show that the *JAK2V617F*-bearing ECs display less cell apoptosis *in vitro* after irradiation compared to *JAK2WT* ECs. In addition, the *JAK2V617F*-mutant Lin<sup>-</sup> HSPCs produce more cells and hematopoietic colonies after irradiation when cultured on *JAK2V617F*-bearing ECs compared to their being cultured on *JAK2WT* ECs. Moreover, the expression levels of CXCL12, EGF, and PTN, which are important niche factors involved in HSPC maintenance and/or HSPC regeneration following radiation injury, were up-regulated in irradiated *JAK2V617F*-bearing ECs compared to *JAK2WT* ECs (Figures 3 and 4). These results suggest that the *JAK2V617F*-bearing vascular niche contribute directly to HSPC radioprotection. Consistent with these findings, 7 of 12 mice in our Tie2/FF1 recipients of normal marrow demonstrated mixed chimerism of an average 77% donor in peripheral blood cells at fourteen weeks following transplantation (Figure 1B). Previously we reported that the *JAK2V617F*-bearing vascular niche promotes the expansion of the *JAK2V617F* HSPCs in preference to *JAK2WT* HSPCs and the development of marrow fibrosis.<sup>20</sup> Since graft failure or poor graft function in MPN patients after SCT is most likely due to marrow fibrosis,<sup>44</sup> our work has demonstrated that the mutant vascular niche can contribute to the poor donor cell engraftment and the high incidence of disease relapse, the two major causes of treatment-related morbidity and mortality associated with allogeneic SCT in patients with MPNs.<sup>2,6,7,44</sup>

The Tie2-Cre mice express Cre recombinase in both ECs and hematopoietic cells. Although an EC-specific Cre (e.g. VEcadherin-Cre) would allow us to distinguish the specific role of ECs in HSPC radioprotection, we chose to

use Tie2-Cre as it mimics the human MPNs in which both the HSPCs and ECs harbor the *JAK2V617F* mutation. In order to determine whether the radioprotection phenotype we have observed in the Tie2/FF1 mice is also due to the *JAK2V617F* mutation in HSPCs, we generated a chimeric murine model with *JAK2V617F*-mutant HSPCs and a WT vascular niche using marrow transplantation. We found that, in the WT vascular niche, the *JAK2V617F*-mutant Lin<sup>-</sup> HSPC is more (not less) sensitive to radiation-induced apoptosis than *JAK2WT* HSPCs. Although there have been reports that EC infusion could augment hematopoietic recovery following myeloablative injury, transplanted ECs exert their pro-regenerative effect transiently, and there is no evidence that donor marrow ECs could engraft and achieve long-term reconstitution in the recipient marrow vascular niche.<sup>31,32,47</sup> Therefore, analysis six weeks post transplantation (Figure 2A and B) is unlikely to be affected by “carry-over” ECs from the Tie2/FF1 donor at the time of transplantation. In addition, the engraftment potential of irradiated *JAK2V617F*-mutant HSPCs does not differ from irradiated *JAK2WT* HSPCs (Figure 2). The results of these studies suggest that the radioprotection phenotype we have observed in the Tie2/FF1 recipients is unlikely to be due solely to the presence of the *JAK2V617F* mutation in HSPCs. In contrast, in another chimeric murine model with WT HSPCs and *JAK2V617F*-bearing vascular niche, WT Lin<sup>-</sup> HSPC is less sensitive to radiation-induced apoptosis in the *JAK2V617F*-mutant vascular niche compared to WT vascular niche (Figure 3). These results suggest that *JAK2V617F*-bearing vascular niche contributes directly to HSPC radioprotection.

We could not exclude the possibility that altered interactions between the *JAK2V617F* HSPCs and *JAK2V617F* ECs contribute to HSPC radioprotection in the Tie2/FF1 mice. Indeed, no significant difference was observed in cell numbers or hematopoietic progenitors between *JAK2WT* HSPCs cultured on *JAK2V617F*-bearing ECs compared to their being cultured on *JAK2WT* ECs after irradiation (Figure 3A and B). This observation suggests that the *JAK2V617F*-bearing vascular niche by itself may not be sufficient to account for the radioprotection phenotype. Rather, it is most likely that specific cell-cell interactions involving the stem cells and niche ECs are required to provide the radioprotection of *JAK2V617F* HSPCs when present in a *JAK2V617F* vascular niche, as exemplified by the up-regulated EGF-EGFR signaling reported in this study (Figure 4). Systemic analysis of HSPC and EC proteins using either quantitative proteomics or antibody-based arrays, along with specific knock-out mouse models would be required to further investigate the interactions between HSPCs and ECs in *JAK2V617F*-bearing MPNs *in vitro* and *in vivo*.

Although the *JAK2V617F* mutation has only been reported in liver and spleen ECs from patients with MPNs,<sup>15,18</sup> it is very probably also present in their marrow ECs, considering that liver, spleen, and marrow are all hematopoietic organs during embryonic development and/or throughout adulthood. In most MPN patients, the stem cell compartment in MPN is heterogeneous with the presence of both *JAK2* wild-type clones and the *JAK2V617F* mutant clones. We hypothesize that the vascular niche in MPN patients is also heterogeneous with the co-existence of both normal and mutant ECs. Since the *JAK2V617F* mutation is present in all HSPCs and ECs



from birth in the Tie2/FF1 mice, our murine model may not present the same acquired clonality and heterogeneous vascular niche features that characterize patients with MPNs. Nonetheless, our study has demonstrated that the JAK2V617F-bearing vascular niche can protect the JAK2V617F HSPCs from the otherwise lethal irradiation administered during conditioning for marrow transplantation, which provides a mechanism for the high incidence of disease relapse in MPN patients after allogeneic SCT. The optimal conditioning regimen for MPN patients undergoing SCT has still not been determined.<sup>44,48</sup> As most current conditioning regimens for SCT are not restricted to only radiation, further investigation using murine models with different quantities of mutant ECs versus WT ECs will be required to fully understand the effects of the JAK2V617F-bearing vascular niche on mutant HSPC

expansion and HSPC resistance to lethal irradiation and the cytotoxic chemotherapies commonly used in SCT for patients with MPNs.

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