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VEGFR-3 is expressed on megakaryocyte precursors in the murine bone marrow and plays a regulatory role in megakaryopoiesis.

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Abstract

Vascular endothelial growth factor receptor (VEGFR) 3 is a transmembrane receptor tyrosine kinase that is activated by its ligands VEGF-C and VEGF-D. Although VEGFR-3 has primarily been linked to the regulation of lymphangiogenesis, we demonstrate here a role for VEGFR-3 in megakaryopoiesis. Using a human erythroleukemia (HEL) cell line and primary murine bone marrow cells, we show that VEGFR-3 is expressed on megakaryocytic progenitor cells through to the promegakaryoblast stage. Functionally, specific activation of VEGFR-3 impaired the transition to polyploidy of $CD41^+$ cells in primary bone marrow cultures. Consistently, blockade of VEGFR-3 promoted endoreplication. *In vivo,* long-term activation or blockade of VEGFR-3 did not significantly affect steady-state murine megakaryopoiesis or platelet counts. However, activation of VEGFR-3 in sub-lethally irradiated mice resulted in significantly elevated numbers of $CD41^+$ cells in the bone marrow and a significant increase in diploid $CD41^+$ cells, whereas the number of polyploid $CD41^+$ cells was significantly reduced. Moreover, activation of VEGFR-3 significantly increased platelet counts in thrombopoietin (TPO)-treated mice, and strongly modulated 5-FU-induced thrombocytosis, suggesting a regulatory role for VEGFR-3 in megakaryopoiesis.

Introduction

VEGFR-3 is a member of the VEGFR receptor tyrosine kinase family. It is expressed on lymphatic endothelial cells (LECs) and plays a central role in the regulation of lymphangiogenesis.[1] Upon binding to its ligands VEGF-C and VEGF-D, VEGFR-3 is activated, and orchestrates the outgrowth of lymphatic vessels.[1]

While the role of VEGFR-3 in regulating lymphangiogenesis is well established[1], several lines of evidence suggest that in addition to being involved in the regulation of lymphangiogenesis, VEGFR-3 may also play a role during hematopoiesis. Targeted deletion of VEGFR-3 in mice results in defective definitive hematopoiesis.[2] Furthermore, VEGFR-3 is also expressed in $CD14^+$ monocytes[3,4] and circulating $CD34^+$ endothelial precursors[5]. Moreover, VEGFR-3 is expressed in human leukemia[6] as well as certain leukemic cell lines[7-9]. Indeed, VEGFR-3 was first identified in HEL cells.[7] With these observations in mind, we investigated whether VEGFR-3 plays a role during hematopoiesis, and found that VEGFR-3 is expressed on megakaryocyte precursor cells in the bone marrow.

During murine hematopoiesis, Sca-1+ hematopoietic stem cells give rise to the precursors of all hematopoietic lineages.[10] Megakaryocytes develop from $CD34^+$ progenitors.[11,12] Proliferation and differentiation of megakaryocyte progenitors is mainly driven by thrombopoietin (TPO), a key regulator of megakaryopoiesis and thrombopoiesis.[13] Upon induction of differentiation, the progenitors pass through several precursor stages, during which time they change from being $CD38^-$ to $CD38^+$ and finally develop into promegakaryoblasts.[14,15] The promegakaryoblasts progressively become polyploid as a result of endoreplication and lose expression of CD34.[12] This process results in the development of mature megakaryocytes that produce platelets.[16] While CD41 and CD61 are expressed during all stages of megakaryocytic differentiation from the progenitor through to the mature megakaryocyte[17,18], CD42 is expressed slightly later during megakaryopoiesis[12]. All three molecules therefore serve as useful markers of this lineage[15,19].

Here we show that VEGFR-3 is expressed on megakaryocyte precursors and during the early endoreplication of promegakaryoblasts, but is not present on mature megakaryocytes. Accordingly, specific activation of VEGFR-3 in primary bone marrow cultures impaired the transition to polyploidy of $CD41^+$ cells, whereas treatment with VEGFR-3 blocking antibodies conversely promoted endoreplication. For the specific activation of VEGFR-3, we used a mutant form of VEGF-C[20] that binds only to VEGFR-3 but not VEGFR-2, which is also present on megakaryocytic cells[21]. While treatment of experimental mice with VEGFR-3-specific ligand or blocking antibodies did not significantly alter steady-state megakaryopiesis or thrombopoiesis, VEGFR-3 activation after sub-lethal irradiation significantly increased the numbers of $CD41^+$ bone marrow cells, and led to a significant decrease in polyploid cells as well as a significant increase in diploid $CD41^+$ cells, consistent with our findings *in vitro*. Moreover, activation of VEGFR-3 in TPO-treated mice significantly increased peak platelet counts, but markedly modulated thrombocytosis in 5-FU-treated animals, suggesting a regulatory role for VEGFR-3 in megakaryopoiesis and subsequent thrombopoiesis.

Materials & Methods

Cell Culture

HEL cells were obtained from DSMZ (Braunschweig, Germany) and cultivated in RPMI (Gibco, Karlsruhe, Germany) containing 10% FCS, 1% penicillin-streptomycin. Differentiation was induced with 10 nM TPA (Sigma, Taufkirchen, Germany). Primary human microvascular LECs (Cambrex, Verviers, Belgium) from the dermis (HMVEC-dLyNeo) were cultivated in EGM-2MV (Lonza, Basel, Switzerland), 5% FCS, supplemented with growth factors provided by the manufacturer. Bovine lymphatic endothelial (BLE) cells were cultivated in DMEM (Gibco) containing 20% FCS and 1% penicillin-streptomycin on gelatin-coated plastic. 293 cells were cultivated in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin.

Western blot analysis

Cell lysates were analysed using standard Western blotting techniques. The membranes were probed with antibodies specific for VEGFR-3 (R&D Systems, Wiesbaden, Germany~~eBioscience, Frankfurt, Germany~~), CD31 (Santa Cruz, Heidelberg, Germany), CD34 (~~Dianova~~Abcam, ~~Hamburg~~Cambridge, ~~Germany~~UK), CD42a (Santa Cruz), CD61 (~~Dako, Hamburg, Germany~~R&D Systems), CD144 (Santa Cruz) or GpA (IBGRL, Bristol, UK). Probing with ~~GATA-1~~HPRT antibodies (Santa Cruz) served as a loading control.

PCR analysis

RNA was prepared using peqGOLD RNAPure (PeqLab, Erlangen, Germany). Synthesis of cDNA using Superscript II (Invitrogen, Karlsruhe, Germany) was performed according to the manufacturer's recommendations. For PCR, cDNAs were amplified as follows: 94˚C, 30 seconds, 60˚C, 30 seconds and 72˚C, 90 seconds (VEGFR-2, Prox1, Lyve1, Podoplanin, hprt, Fli-1, Fog-2, Gata-2 and Elf-1) or 94˚C, 30 seconds, 54˚C, 30 seconds and 72˚C, 90 seconds (VEGFR-3). Details of the primers used are in supplementary Material and Methods.

Tubule formation on collagen gels

Collagen type 1 was prepared from rat tails. Tendons were isolated, dissolved in acetic acid, then filtered, lyophilized and re-dissolved in 0.1% acetic acid at 4 mg/ml. Cells were seeded on collagen gels (2 mg/ml) and cultured in the presence of 30 ng/ml $VEGF_{165}$ (Promokine, Heidelberg, Germany) for 8 days. Tubule formation was analysed as previously described.[22]

IHC

For the immunohistochemical analysis of VEGFR-3 expression in the bone marrow, cryosections of de-calcified murine femurs embedded in tissue freezing medium (Leica, Nussloch, Germany) were fixed in acetone and stained with VEGFR-3 antibodies (eBioscience).

MACS

Bone marrow cells isolated from femurs and tibiae of C57BL/6 mice were treated with Fc-block (Becton Dickinson, Heidelberg, Germany), then incubated with antibodies against VEGFR-3 (R&D Systems~~, Wiesbaden, Germany~~), Sca-1, CD41 or CD38 (all Becton Dickinson) followed by specific secondary MACS antibodies (Miltenyi, Bergisch-Gladbach, Germany) according to the manufacturer´s recommendations. Cell populations were then either enriched or depleted for the labelled epitope using LS or LD columns (Miltenyi), respectively. The purity of the sorted populations was controlled by flow cytometry.

CD42 FACS

Bone marrow was isolated from femurs and tibiae of C57BL/6 mice, then stained with antibodies specific for VEGFR-3 (R&D Systems) and/or CD42a (Emfret, Eibelstadt, Germany), and analysed in FACS.

Lethal irradiation and bone marrow transplantation

C57BL/6 mice were irradiated with lethal doses (9 Gy) from a γ-source. After 24 hours, the mice were all transplanted in parallel by intravenous injection with either complete

bone marrow, bone marrow depleted of VEGFR-3 positive cells or bone marrow mock depleted with an appropriate isotype control using MACS as described above. EDTA blood samples were taken from all animals on days 0, 5, 7, 9, 12, 15, 19, 22, 26, 29, 33 and 44 post transplantation, and analysis was performed by a commercial veterinary laboratory (Laboklin, Bad Kissingen, Germany).

Isolation and culture of primary murine bone marrow cells

Bone marrow was isolated from femurs and tibiae of C57BL6 mice. After lysis of red blood cells with ammonium-chloride-potassium (ACK) buffer, the cells were transferred to IMDM (Gibco) supplemented with 1% penicillin/streptomycin, 10% 293 cell-conditioned DMEM, Nutridoma SP (Roche, Mannheim, Germany), L-glutamine and 100 pg/ml recombinant murine thrombopoietin (RDI Diagnostics, Concord, USA). Depending on the experiment the cells were cultured with either 100 µg/ml mF4-31C1 VEGFR-3 blocking antibodies (kindly provided by ImClone Systems, New York, USA), 100 µg/ml of rat IgG isotype control or 400 ng/ml of VEGF-C-Cys, a mutant form of VEGF-C that activates VEGFR-3 but not VEGFR-2[20]. After 72 hours, the medium was renewed, and supplemented with 37 ng/ml thrombopoietin. After a further 72 hours, the cells were harvested, treated with Fc-block (Becton Dickinson), incubated with CD41 antibodies, and fixed in 70% EtOH. Nuclei were stained with Draq5 (Biostatus Ltd, Shepshed, UK), then the cells were analysed using a FACScan cytometer (Becton Dickinson). The bone marrow from each animal was either divided equally, each half receiving either VEGF-C-Cys or control treatment, or undivided bone marrow was used for the treatments (VEGFR-3 blocking). Significance was therefore tested accordingly using two-tailed paired or unpaired t-tests, respectively.

Long-term injections

C57BL/6 mice were injected daily with 25 µg VEGF-C-Cys for three weeks. Blood was taken on days 0, 3, 7, 10, 14, 17 and 21. In the blocking antibody experiments, mice were injected with 600 µg/animal per injection of mF4-31C1 VEGFR-3 blocking antibody, isotype control Ig, or PBS, following a Monday-Wednesday-Friday schedule for six weeks. Blood was taken on days 0, 8, 13, 16, 20, 23, 27, 30, 34, 37, 41 and 44, and

analysed as described above. In each experiment, all animals were treated at the same time and on the same day, and all animals were bled at each time point. At the end of the experiment, bone marrow was isolated from femurs and tibiae, treated with Fc-block (Becton Dickinson), stained with FITC-conjugated anti CD41 antibodies (Becton Dickinson), then fixed in 70% EtOH. Nuclei were stained with Draq5 (Biostatus Ltd, Shepshed, UK), and the number and ploidy of CD41 positive cells was subsequently analysed using a FACScan cytometer (Becton Dickinson). Significance was tested using two-tailed non-paired t-tests, assuming equal variance.

Recovery kinetics after sub-lethal irradiation

Experimental C57BL/6 mice were sub-lethally irradiated (4.5 Gy) in a γ-source. They were then either injected daily with VEGF-C-Cys (25 µg/animal and injection) or PBS, or were injected with 600 µg/animal per injection of mF4-31C1 VEGFR-3 blocking antibodies, isotype control Ig or PBS every other day intra-peritoneally. Blood was taken on days 0, 7, 11, 14, 18 and 21 post irradiation and analysed as described above. In each experiment, all animals were treated at the same time and on the same day, and all animals were bled at each time point. Bone marrow was isolated from femurs and tibiae 20 days after irradiation, and the number and ploidy of CD41 positive cells in the bone marrow was assessed as described above. Significance was tested using two-tailed non-paired t-tests, assuming equal variance.

TPO administration

C57BL/6 mice were administered with 5 µg recombinant murine TPO (RDI), followed by daily injections of either 25 µg VEGF-C-Cys or PBS. One group received only PBS throughout. Blood was taken and analysed 0, 3, 5, 7 and 10 days post TPO administration. All animals were treated at the same time and on the same day, and all animals were bled at each time point. After 10 days, the animals were killed and the number and ploidy of CD41 positive cells in the bone marrow was assessed as described above. Significance was tested using two-tailed non-paired t-tests, assuming equal variance.

5-FU treatment

C57BL/6 mice were injected intra-peritoneally with a single dose of 5-FU (Sigma) at 150 mg/kg. Control mice remained untreated. The 5-FU-treated mice then received daily injections of either 25 μg VEGF-C-Cys or PBS throughout the experiment. Blood was taken and analysed 0, 5, 8, 12, 16, 19, 24, 29, 33, 38, 43, 47 and 52 days post 5-FU administration. All animals were treated at the same time and on the same day. Significance was tested using two-tailed non-paired t-tests, assuming equal variance.

All animal experiments were approved by the local authorities, and performed according to the German legal requirements.

Results

Expression of VEGFR-3 and other lymphatic endothelial markers is upregulated upon phorbol diester-induced megakaryocytic differentiation of HEL cells.

VEGFR-3 is widely used as a marker for lymphatic endothelium. Originally, however, the receptor was cloned from the human erythroleukemia cell line HEL.[7] This cell line can be induced to differentiate into the erythrocyte lineage upon erythropoietin (EPO) treatment[23] and into the megakaryocyte lineage in response to tetradecanoyl phorbol acetate (TPA)[24]. We therefore decided to examine expression of VEGFR-3 during differentiation of HEL cells. No change in VEGFR-3 expression was observed upon EPO treatment (data not shown). However, VEGFR-3 expression was dramatically upregulated after 72 hours treatment with TPA (Figure 1A).

~~Consistent~~ In line with the notion that HEL cells differentiate into the megakaryocyte lineage upon TPA treatment, we detected strong upregulation of a number of markers and transcription factors associated with megakaryocytic differentiation (Figure 1B, C).~~,~~ Consistent with previous findings[24], ~~although~~ we also found that expression of Glycophorin A whose expression is associated~~, usually correlated~~ with erythroid differentiation, was ~~also found to be increased~~not reduced upon TPA-induced megakaryocytic differentiation (Figure 1B). S~~, which is,~~. h~~However, consistent with previous findings, which show that the Glycophorin A protein is not downregulated upon TPA-induced megakaryocytic differentiation of HEL cells.~~[24] ~~Upon~~ubsequent to TPA treatment, both adherent and suspension populations of HEL cells were observed that exhibited virtually identical marker profiles and viability. Apart from their adhesive properties and the expression levels of fog-2~~, which was higher in the adherent population~~, and CD144, we were unable to distinguish between them (Figure 1 B, C; data not shown).

A survey of the literature revealed that virtually all markers described to date as being expressed on megakaryocytes can also be expressed on endothelial cells (Supplementary

Figure ~~2~~1). This prompted us to examine whether other genes typical of LECs in addition to VEGFR-3 are also upregulated in HEL cells upon TPA treatment. Indeed, we found that Prox-1 and LYVE-1, markers of lymphatic endothelium, are also upregulated after TPA treatment of HEL cells (Figure 1D).

These observations raised the question whether HEL cells really undergo megakaryocytic differentiation after TPA treatment, or whether they adopt an endothelial phenotype with LEC characteristics. To address this point, we tested whether TPA-treated HEL cells are capable of forming capillaries, reasoning that if the cells differentiated into endothelial cells, this should be the case. However, in contrast to control bovine LECs, TPA-treated HEL cells could not be induced to form capillaries (Figure 2A). Furthermore, multinucleated structures typical of megakaryocyte differentiation were observed in TPA-treated HEL cells (Figure 2B), and TPA treatment induced limited polyploidy in HEL cells (Figure 2C), again pointing to megakaryocytic differentiation.

Together, these data show that expression of VEGFR-3 and other markers typical of LECs is induced when HEL cells differentiate into the megakaryocytic lineage.

VEGFR-3 is expressed on megakaryocytic progenitors through to the pro-megakaryoblast stage in the bone marrow.

The upregulation of VEGFR-3 during HEL cell megakaryoctic differentiation suggested to us that VEGFR-3 may play a role in megakaryopoiesis. Due to the limited megakaryocytic differentiation capacity of HEL cells and their cancerous nature, we explored this possibility further using murine bone marrow.

First we characterised VEGFR-3 expression in the bone marrow. FACS staining revealed that around 2% of murine bone marrow cells are VEGFR-3-positive (Figure 3A). Furthermore, when sections of bone marrow were immunologically stained with anti-VEGFR-3, isolated mononuclear VEGFR-3$^+$ cells were observed, but mature

megakaryocytes were VEGFR-3$^-$ (Figure 3B). DNA profiling of VEGFR-3$^+$ bone marrow cells revealed that most have a ploidy of 2n and 4n, with fewer numbers (around 10%) of 8n cells (Figure 3 C and Supplementary Figure ~~3~~2). VEGFR-3+ cells with polyploidy greater than 8n were not observed.

To define further the stages of megakaryopoiesis during which VEGFR-3 is expressed, co-stainings with the stem cell marker Sca-1, as well as CD38, CD41 and VEGFR-3 were performed. Expression of Sca-1 is lost during myeloid differentiation.[25] CD38 expression, in turn, is increased early in megakaryopoiesis from the BFU-MK stage on.[26] Finally, CD41 becomes substantially upregulated at the beginning of endoreplication and subsequent polyploidy.[15,18] The double stainings showed that 1.8% of the Sca-1$^+$, 5.1% of the CD38$^+$ and 3% of CD41$^+$ cells were also VEGFR-3$^+$ (Table 1 and Supplementary Figure ~~4~~3).

These observations suggested to us that VEGFR-3 might be expressed on hematopoietic stem cells through to the promegakaryoblast stage. However, Sca1 is not only expressed on hematopoietic stem cells but also on the immediate progenitors arising from the stem cells.[27] In order to test whether VEGFR-3 is expressed on hematopoietic stem cells, we transplanted either complete, or VEGFR-3-depleted bone marrow into lethally irradiated mice. The recovery of thrombocytes and erythrocytes was monitored. As expected, circulating platelet counts dropped dramatically the first few days after the irradiation, and later recovered (Figure 3D). However, we did not observe any difference in the kinetics of recovery between the groups transplanted with VEGFR-3 depleted bone marrow and the control groups.

Together, these data are consistent with the notion that VEGFR-3 is not expressed on hematopoietic stem cells, but rather on megakaryocyte precursors through to the pre-megakaryoblast stage, and that VEGFR-3 expression is lost as megakaryocytes further mature. This notion is further substantiated by the observation that VEGFR-3+ bone marrow cells co-express CD42, a marker for megakaryocytes that is not expressed on hematopoietic precursor cells (Supplementary Figure ~~5~~4).[28]

We also examined whether megakaryocytic lineage cells express LEC markers, similar to the situation in HEL cells. This proved to be the case, as $CD41^+$ cells expressed Lyve-1 (Supplementary Figure ~~6A~~5A), and the majority of VEGFR-3+ bone marrow cells express podoplanin (Supplementary Figure ~~6B~~5B).

Manipulation of VEGFR-3 influences megakaryopoiesis *in vitro*.

In order to examine the role that VEGFR-3 plays during megakaryopoiesis, we cultivated primary murine bone marrow cells with physiological concentrations of thrombopoietin (TPO) to maintain the megakaryocyte precursors. The cells were grown for three days in the presence or absence of VEGF-C-Cys, a mutant form of VEGF-C that specifically activates VEGFR-3 but not VEGFR-2[20], because VEGFR-2 is also present on megakaryocytic cells[21]. The cells were then incubated for a further three days in the presence of saturating concentrations of TPO to drive them into megakaryocytic differentiation. The cultured cells were subsequently stained with anti-CD41 antibodies to mark the megakaryocytic population and with Draq5 for DNA profiling. FACS analysis revealed that VEGF-C-Cys treatment significantly increased the number of 2n $CD41^+$ cells in the cultures ($p=0.019$), while the number of $CD41^+$ cells with a ploidy greater than 4n were concomitantly reduced ($p=0.047$) (Figure 4A). In a converse experiment, we inhibited VEGFR-3 activation with a specific blocking antibody (Supplementary Figure ~~7~~6), and observed that the number of 2n $CD41^+$ cells significantly decreased ($p=0.012$), while the number of polyploid (>4n) $CD41^+$ cells was significantly elevated ($p=0.014$) upon blocking of VEGFR-3 activation (Figure 4B). Treatment with VEGF-A, which activates VEGFR-2, and chimeric VEGFR-2 receptor globulins served as additional controls. However, neither treatment with VEGF-A, nor incubation with chimeric VEGFR-2 receptor globulins significantly affected the ploidy distribution of cultured primary $CD41^+$ bone marrow cells (Supplementary Figure ~~8~~7).

Our data suggest that the specific activation of VEGFR-3 during megakaryopoiesis impairs the transition to polyploid stages, whereas blocking the receptor promotes

differentiation and endoreplication.

Neither activation nor blocking of VEGFR-3 influences steady-state megakaryopoiesis and thrombopoiesis *in vivo*.

To study potential effects of VEGFR-3 manipulation on megakaryopoiesis and thrombopoiesis *in vivo*, we first injected VEGF-C-Cys to activate VEGFR-3, or PBS as a control into mice on a daily basis for three weeks. Thrombocyte concentrations in the blood were monitored regularly. After three weeks of treatment, the mice were killed. Bone marrow cells were isolated and stained for CD41 and DNA content in order to evaluate the number and ploidy of the $CD41^+$ population. We observed a significant decrease in apoptotic $CD41^+$ bone marrow cells in the VEGF-C-Cys treated group ($p<0.01$), and a trend towards reduced polyploidy as well as an increase in 2n $CD41^+$ cells, analogous to our *in vitro* observations. VEGF-C-Cys had no effect on platelet counts or the number of $CD41^+$ cells in the bone marrow (Supplementary Figure ~~9~~ 8 A-C).

To test the effect of inhibiting VEGFR-3 activation on megakaryopoiesis and thrombopoiesis *in vivo,* mice were injected daily with VEGFR-3 blocking antibodies or an appropriate isotype control for six weeks. Platelet counts were monitored regularly and the numbers as well as ploidy distribution of $CD41^+$ bone marrow cells were analysed at the end of the experiment. Under these conditions, no effects on the measured parameters were observed (Supplementary Figure ~~9~~ 8 D-F).

Activation of VEGFR-3 increases platelet counts in TPO-stimulated animals, modulates 5-FU-induced thrombocytopenia and thrombocytosis, and influences ploidy distribution and numbers of $CD41^+$ bone marrow cells after sub-lethal irradiation.

Thrombocyte homeostasis is tightly controlled in mammals, and alternative mechanisms exist that can compensate for perturbation of particular regulatory pathways.[13] As we observed significant effects of VEGFR-3 manipulation on primary murine bone marrow cells *in vitro* but not *in vivo*, we reasoned that this might be due to such compensatory mechanisms. To circumvent this, we manipulated VEGFR-3 activation under conditions in which megakaryopoiesis and thrombopoiesis are stimulated over and above the normal physiological steady-state.

First, we examined whether VEGFR-3 activation affects TPO-induced megakaryopoiesis and thrombopoiesis. Administration of a single dose of TPO leads to a dramatic temporary increase in platelet counts that peaks at day five, then decreases to physiological concentrations within ten days post TPO administration.[29] In order to test whether VEGFR-3 activation affects TPO-mediated effects on platelet production, we injected mice with an initial dose of TPO, followed by daily administration of VEGF-C-Cys. We found that VEGF-C-Cys significantly increased peak platelet counts at day five in comparison with the control group (Figure 5A).

In rodents, 5-FU is known to induce thrombocytopenia, followed by pronounced thrombocytosis, which can occur in waves and has been shown to occur independently of TPO.[30] We injected mice with a single dose of 5-FU and the animals were subsequently treated with VEGF-C-Cys. We found that VEGFR-3 activation transiently, but significantly ($p<0.002$) limited 5-FU-induced thrombocytopenia, as well as thrombocytosis ($p=0.005$) in comparison with the control group (Figure 5B). Furthermore, VEGFR-3 activation led to a more sustained thrombocytosis compared to the control group ($p<0.04$) during the second wave of thrombocytosis (Figure 5B).

Finally, we sub-lethally irradiated mice to partially destroy the bone marrow, then followed the recovery of $CD41^+$ bone marrow cells. VEGFR-3 activity was manipulated by injecting the mice with either VEGF-C-Cys or VEGFR-3 blocking antibodies. Thrombocyte numbers were not significantly affected by these treatments (Figure 5 C, F). However, activation of VEGFR-3 via VEGF-C-Cys injection led to a significant increase

in $CD41^+$ cells (Figure 5H). Furthermore, the number of 2n $CD41^+$ bone marrow cells was significantly increased, whereas the number of polyploid cells was significantly lower than in the control group (Figure 5G), matching the original findings made *in vitro* and the trend observed in non-irradiated mice *in vivo*.

Discussion

Here we report that in addition to its well-studied expression on LECs, VEGFR-3 is also expressed on megakaryocyte progenitors and early promegakaryoblasts. Activation of VEGFR-3 in primary bone marrow cultures and in sublethally irradiated mice significantly reduced the number of polyploid $CD41^+$ cells, whereas the number of diploid $CD41^+$ cells was significantly elevated. Additionally, the total number of $CD41^+$ bone marrow cells was substantially increased upon VEGFR-3 activation in the irradiated mice. Furthermore, a significant increase in platelet numbers over and above that observed with TPO alone was obtained as a consequence of VEGFR-3 activation in TPO-treated mice. In addition, 5-FU-induced thrombocytopenia and thrombocytosis were substantially modulated by VEGFR-3 activation. Together these data suggest that the VEGFR-3 expressed on the megakaryocytic lineage plays a regulatory role in megakaryopoiesis and thrombopoiesis.

We found that VEGFR-3 is co-expressed with Sca-1, a marker of hematopoietic stem and precursor cells, and with CD41, a megakaryocytic marker that has also been reported to be expressed by hematopoietic precursor cells with myeloid and lymphoid potential.[28] Although these findings suggest that hematopoietic stem cells might express VEGFR-3, we did not observe any changes in the recovery kinetics of thrombocytes and erythrocytes in lethally irradiated mice transplanted with VEGFR-3-depleted bone marrow compared to those transplanted with complete bone marrow (Figure 3D). A difference would be expected if the $VEGFR\text{-}3^+$ subpopulation contains early pluripotent precursors or stem cells. In addition to the observed expression of CD41, several other of our observations strongly support the notion that VEGFR-3 is expressed on megakaryocytic progenitors and promegakaryoblasts. For example, we found that CD42, a surface protein that is absent from hematopoietic precursor cells[28] but is expressed during megakaryopoiesis[12], is co-expressed with VEGFR-3 on bone marrow cells (Supplementary Figure ~~5~~4). Furthermore, a fraction of $VEGFR\text{-}3^+$ cells in the bone marrow is polyploid, a hallmark of megakaryocytes unique in the bone marrow (Figure 3C).

Our data suggest that cells in the megakaryocytic lineage express several genes commonly used as markers of lymphatic endothelial cells. Specifically, we found that HEL cells differentiate into the megakaryocytic lineage upon TPA stimulus and upregulate VEGFR-3, LYVE-1 and Prox-1. In the murine bone marrow, we found LYVE-1 to be expressed by both $CD41^-$ and $CD41^+$ cells (Supplementary Figure ~~6A~~5A). While LYVE-1 is known to be expressed by macrophages in the $CD41^-$ population[31], the $CD41^+$ $LYVE\text{-}1^+$ cells likely represent a megakaryocytic population. Furthermore, FACS analysis also showed that the lymphatic marker podoplanin is expressed by the majority of VEGFR-3+ bone marrow cells (Supplementary Figure ~~6B~~5B). Similarities between endothelial and myeloid cells in terms of their expression profiles (Supplementary Figure ~~2~~1) have been described earlier.[32,33] One reason for this similarity may be a common precursor cell, the hemangioblast, that is believed to give rise to both lineages.[34]

Several of the experiments reported here suggest that changes in megakaryopoiesis do not necessarily correspond directly to altered thrombocyte numbers. For example, we found that platelet counts returned to normal after TPO treatment (Figure 5A), but that the number of $CD41^+$ cells from TPO-treated animals remained high compared with non-treated controls, and their ploidy distribution was also affected (Supplementary Figure ~~10~~9), supporting the notion that platelet counts do not necessarily reflect the number and ploidy distribution of $CD41^+$ cells. Furthermore, VEGF-C-mediated VEGFR-3 activation in sub-lethally irradiated mice increased the number of $CD41^+$ cells and suppressed the development of polyploidy, but had no significant impact on thrombopoiesis (Figure 5). Although we cannot completely exclude that the substantial increase in $CD41^+$ cells observed upon VEGFR-3 activation may have balanced out the reduction in polyploid cells and thus kept thrombocyte numbers at similar levels to the controls, these data nevertheless also speak for independent regulation of megakaryopoiesis and thrombopoiesis. This notion is supported by a number of studies that show that TPO levels do not always correspond to platelet counts.[13]

The inhibition of the development of polyploidy in $CD41^+$ cells we observed upon

VEGFR-3 activation *in vitro* and after treatment of irradiated animals with VEGF-C-Cys has parallels with the regulation of megakaryopoiesis by TPO and TGF-β. TGF-β, a negative regulator of megakaryopoiesis, is stored in thrombocytes in high amounts. It is released when thrombocytes disintegrate and induces TPO expression in bone marrow stromal cells. TPO in turn upregulates TGF-β receptor expression on megakaryoblasts, thereby repressing unlimited TPO-mediated maturation of CFU-Meg.[35] Elevated serum TGF-β levels therefore result in increased TPO–induced differentiation of hematopoietic stem cells into the megakaryocytic lineage, but TGF-β-mediated repression of maturation beyond the CFU-Meg stage results in an accumulation of CFU-Megs. As TGF-β levels decline, repression is released and a temporarily increased number of megakaryocytes replenish the thrombocyte pool.[35] On the basis of the results presented here it is tempting to speculate that VEGF-C may act in concert with TPO/TGF-β to first repress endoreplication, leading to an increased number of 2n megakaryocytic precursors, followed by release of repression and promotion of thrombopoiesis. Pathophysiological contexts exist where this scenario seems plausible. For example, VEGF-C is upregulated during *in vitro* megakaryopoiesis[36], is found in thrombocytes and is released upon platelet activation[37], similar to TGF-β. TPO and VEGF-C are also both induced by IL-6.[1,38] Notably, both TPO and TGF-β levels increase after irradiation[39,40], and could therefore also combine with VEGFR-3 activation to exert the effects on megakaryopoiesis we observed here upon sub-lethal irradiation.

VEGFR-3 activation may also act to regulate megakaryocyte differentiation and platelet production independently of TPO. While TPO is the principal regulator of megakaryocytic proliferation and differentiation, other factors such as interleukins can influence megakaryopoiesis and thrombopoiesis. Some of these factors (e.g. IL-6) probably act indirectly by inducing TPO expression.[13] Nevertheless, targeted deletion of the TPO receptor c-Mpl in mice does not completely destroy megakaryopoiesis and thrombopoiesis[41] indicating additional TPO-independent control mechanisms. It is therefore significant that c-mpl deficient mice respond to 5-FU treatment with marked thrombocytosis, suggesting that 5-FU-induced thrombocytosis is independent of TPO.[30] Our data shows that 5-FU-induced thrombocytosis is significantly impaired by VEGFR-3

activation, suggesting that VEGFR-3 also plays a role in the TPO-independent regulation of thrombocytosis. In this context, VEGFR-3 activation could potentially act by impairing megakaryocytic differentiation, leading to a reduction in thrombocytosis. Alternatively or in addition, the precursor pool might be expanded, and could potentially contribute to the increased platelet counts we observed during the second wave of thrombocytosis.

VEGFR-3 signalling has been implicated in a number of disease processes, and manipulation of VEGFR-3 activity is a promising therapeutic avenue.[1,42] Therapeutic activation of VEGFR-3 on LECs has been proposed as a treatment for lymphedema.[1] Conversely, VEGF-C/D-regulated, tumor-induced lymphangiogenesis promotes metastasis, and there has been much interest in the potential of VEGFR-3 blockade as a means of suppressing metastasis in the context of tumors.[42,43] Potential side effects of the manipulation of VEGFR-3 activity remain to be investigated. The data presented here suggest that altered megakaryopoiesis and platelet production might occur upon VEGFR-3 manipulation, depending on the context. While we did not observe significant effects in healthy animals, an influence on thrombopoiesis cannot be excluded if TPO levels are significantly elevated, as is the case in patients with thrombocytopenia as a consequence of high dose chemotherapy.[44] Our data also demonstrate that regulation of VEGFR-3 activity in the context of 5-FU treatment can affect thrombopoiesis independently of TPO. Thus a combination of chemotherapy with anti-VEGFR-3 therapy may result in changes in platelet levels not observed with either therapy alone. We note that a number of multi-kinase inhibitors are currently in clinical trials that inhibit VEGFR-3 activation[45], suggesting that platelet levels should be carefully monitored if combination therapies are attempted. Nevertheless, our data also suggest that megakaryopoiesis is more sensitive to VEGFR-3 activation than VEGFR-3 blockade. In contrast to VEGFR-3 activation via VEGF-C-Cys, which showed significant effects on megakaryocytic differentiation *in vitro*, as well as *in vivo*, the administration of VEGFR-3 blocking antibodies significantly affected megakaryopoiesis only *in vitro*, with the exception that, divergent from our *in vitro* findings, the administration of VEGFR-3 blocking antibodies to sublethally irradiated mice led to a significant increase of the 2n $CD41^+$ population. This was,

however, only seen in comparison with the isotype control, but not with the PBS-treated animals (Figure 5D). Also, the polyploid $CD41^+$ population was not affected (Figure 5D). Importantly, we used equivalent doses of the mF4-31C1 VEGFR-3 blocking antibody *in vivo* to those applied in other studies in which the antibody inhibited lymphangiogenesis in experimental animals.[46,47] These data therefore suggest that blockade of VEGF-3 activity at the minimal doses that suppress lymphangiogenesis are unlikely to elicit side effects on platelet levels.

In summary, we demonstrate here a novel role for VEGFR-3 in regulating the megakaryocytic lineage. Further efforts beyond the scope of this study will focus on elucidating the molecular pathways through which VEGFR-3 activation interfaces with the signalling pathways that control megakaryopoiesis and thrombopoiesis. This will also contribute to our understanding of the physiological and pathophysiological contexts in which VEGFR-3 exerts an effect on megakaryopoiesis.

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Authorship Contributions.

WT: coordinated the project, designed and performed research, collected, analyzed and interpreted data, wrote the manuscript **JK:** performed research, collected data, analyzed and interpreted data **MR:** performed research and contributed vital reagents **DW:** performed research, collected and analyzed data **DP:** performed research, collected and analyzed data **VK:** performed research and collected data **LQ:** contributed vital reagents **HAW:** contributed vital reagents **JPS:** designed and coordinated the project, designed research, analyzed and interpreted data, wrote the manuscript.

Conflict of Interest Disclosures.

The authors have no conflict of interest to declare.

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Tables.

Population	Sub-population	Sub-population as % of population +/-SE
Sca-1$^+$	VEGFR-3$^+$ CD41$^+$	0.0 +/- 0.0
Sca-1$^+$	VEGFR-3$^+$ CD41$^-$	1.8 +/- 0.5
Sca-1$^-$	VEGFR-3$^+$ CD41$^+$	0.5 +/- 0.3
Sca-1$^-$	VEGFR-3$^+$ CD41$^-$	0.4 +/- 0.2
CD41$^+$	VEGFR-3$^+$	3.0 +/- 0.5
CD41$^-$	VEGFR-3$^+$	2.5 +/- 1.4
CD38$^+$	VEGFR-3$^+$	5.1 +/- 3.8
CD38$^-$	VEGFR-3$^+$	0.3 +/- 0.3

Table 1: Coexpression of VEGFR-3 with Sca-1, CD38 and CD41 in the murine bone marrow. Bone marrow cells were isolated, and populations positive and negative for Sca-1, CD41, or CD38, respectively, were enriched via MACS (first column of table). The enriched CD41$^+$, CD41$^-$, CD38$^+$ and CD38$^-$ populations were then further stained with VEGFR-3 specific antibodies, whereas the enriched Sca-1$^+$ and Sca-1$^-$ populations were further stained with CD41 and VEGFR-3 specific antibodies (second column of table). Flow cytometry was used to evaluate the percentage of the sub-populations indicated in column two as a fraction of the populations indicated in column one. (n=3). For representative plots and more detailed description of the experimental procedure see Supplementary Figure 43.

Figure legends.

Figure 1: Lymphatic and hematopoietic markers are upregulated in HEL cells upon TPA treatment.

A: VEGFR-3 is upregulated in HEL cells upon TPA treatment. HEL cells were treated with TPA. The cells were harvested 0, 24, 48 or 72 hours after TPA stimulus and analysed for VEGFR-3 expression in Western Blot. Membranes were probed with ~~GATA-1~~HPRT antibodies as a loading control. ~~GATA-1 is not regulated in HEL cells under the conditions used (Supplementary Figure 1), and was therefore used as a loading control.~~ **B:** Regulation of different hematopoietic markers in HEL cells upon TPA treatment. HEL cells were cultured for 72 hours in the presence or absence of TPA. Lysates of the untreated, as well as adherent (ad) and suspension (s) populations occurring after TPA treatment, were analysed in Western blots. Membranes were probed with ~~GATA-1~~HPRT antibodies as a loading control. **C:** Regulation of megakarycytic transcription factors in HEL cells upon TPA treatment. HEL cells were incubated with or without TPA for 72 hours. RNA was isolated from untreated as well as adherent (ad) and suspension (s) populations of treated HEL cells, and transcribed into cDNA. Expression of several transcription factors involved in megakaryopoiesis was assessed using semi-quantitative PCR. Hprt served as a loading control. **D:** Regulation of lymphatic markers in HEL cells upon TPA treatment. HEL cells were incubated with TPA for 72 hours or were left untreated. RNA was isolated from untreated as well as adherent (ad) and suspension (s) populations of treated HEL cells, then transcribed into cDNA, which served as a template for semi quantitive PCR. Human lymphatic endothelial cells were used as a positive control. Amplification of hprt served to demonstrate equal loading.

Figure 2: HEL cells show a megakaryocytic phenotype upon TPA treatment.

A: HEL cells do not form tubule-like structures on collagen**.** HEL cells (left panel) and BLE cells, which served as a positive control (right panel), were grown on collagen gels in the presence of 30 ng/ml $VEGF_{165}$. Scale bars: 200 µm. **B:** TPA treatment induces a polynucleated phenotype in HEL cells. HEL cells were cultivated on a collagen coated

surface in the presence of TPA, ethanol as a solvent control, or were left untreated. After eight days, the cells were fixed and H&E stained. Scalebars: 400 μm (controls); 200 μm (TPA treatment). **C:** TPA induces polyploidy in HEL cells. HEL cells were or were not treated with TPA for 72 hours, harvested, then fixed and their DNA stained with Draq 5. Ploidy of untreated (left panel) and TPA treated (right panel) HEL cells was assessed flow cytometrically.

Figure 3: VEGFR-3 is expressed in the murine bone marrow on early megakaryocytic progenitor cells through to the magakaryoblast stage.

A: VEGFR-3 is expressed on primary murine bone marrow cells. Murine bone marrow cells were isolated, treated with Fc-block and stained with antibodies specific for VEGFR-3 (left panel) or an appropriate isotype control (right panel). FACS analysis showed that 1.85% +/- 0.31% SE (n=9) of the murine bone marrow cells express VEGFR-3. Dot plots of one representative experiment are depicted. Density plots were used to define a region in which 95 % (the two outer contours) of the negative control events were excluded. The region was then applied to a plot displaying the stained sample. The number of positive events in both negative control and the actual sample was then assessed. The percentage of true positive cells was calculated by subtraction of the number of events in the negative control within the defined region from the number of events found in the same region for the actual sample. Identical numbers of events were acquired. **B:** VEGFR-3 is expressed on isolated mononuclear cells in the murine bone marrow. Sections of murine femurae were stained with VEGFR-3 specific antibodies (left panel: VEGFR-3; right panel: control). MK: megakaryocyte; scale bars: 100 μm. **C:** Ploidy of VEGFR-3 positive cells in the murine bone marrow. VEGFR-3 positive bone marrow cells were enriched by MACS and then analysed in FACS. As a control, cells were treated with an appropriate isotype control. Clumping cells mimicking polyploidy were excluded from the analysis by appropriate gating strategies. The resulting histogram plot shows the DNA content of VEGFR-3 positive cells. Dot plots of the DNA content of the cells were used for the quantification of VEGFR-3 positive and isotype treated cells within different ploidy classes or cell cycle stages, respectively (A detailed scheme of the

gating strategy is to be found in Supplementary Figure ~~3~~2). Subtraction of the background signal, generated by unspecific binding of the isotype control, allows the calculation of an actual ploidy distribution within the VEGFR-3 positive population. **D:** VEGFR-3 depletion has no influence on the recovery kinetics of thrombocytes and erythrocytes in lethally irradiated mice. Mice were irradiated with lethal doses of 9 Gy. Within 24 hours after irradiation, the animals were transplanted with VEGFR-3 MACS depleted bone marrow, or complete bone marrow as a control. Blood counts were performed regularly and the recovery of thrombocytes (left panel) and erythrocytes, as a control (right panel) were monitored. (n=5; error bars represent SE).

Figure 4: Influence of VEGFR-3 manipulation on the ploidy of primary murine bone marrow cultures *in vitro*. A: VEGFR-3 activation. Bone marrow cells were cultivated with physiological concentrations of TPO, and in presence or absence of VEGF-C-Cys for 72 hours. The TPO concentration was then increased to 37 ng/ml, and the cells were incubated for a further 72 hours, after which they were harvested, stained with CD41 specific antibodies, and fixed. After DNA staining with Draq 5, the $CD41^+$ cells were analysed for their DNA content. The upper plot shows the ploidy distribution of $CD41^+$ bone marrow cells. (n=6; error bars represent SE; *: $p<0.05$). Histogram plots of representative FACS results are presented below. **B:** VEGFR-3 blocking. Bone marrow cells were cultivated in the presence of physiological concentrations of TPO, and VEGFR-3 blocking antibodies or an appropriate isotype control for 72 hours. The TPO concentration was then increased to 37 ng/ml and the cells were incubated for a further 72 hours, after which they were harvested, stained with CD41 specific antibodies, and fixed. After DNA staining with Draq 5, the $CD41^+$ cells were analysed for their DNA content. The upper plot shows the ploidie distribution of $CD41^+$ bone marrow cells. (n=10; error bars represent SE; *: $p<0.05$). Histogram plots of representative FACS results are presented below.

Figure 5: Effects of VEGFR-3 manipulation on platelet counts in TPO-stimulated

and 5-FU-treated mice, and on platelet recovery and CD41$^+$ bone marrow cells after sub-lethal irradiation *in vivo*. A: Mice were either mock treated or stimulated with an initial dose of TPO. The mock-treated animals then received daily PBS injections (PBS/PBS). The TPO treated mice received daily injections of either VEGF-C-Cys (TPO/VEGF-C-Cys) or carrier (TPO/PBS) as a control for 10 days, during which time platelet counts increased then recovered. Blood was taken regularly and platelet counts were performed. (n=8; error bars represent SE; *: p=0.05). **B:** Mice were administered with a single dose of 150 mg/kg 5-FU and thereafter received daily injections of either VEGF-C-Cys (5-FU/VEGF-C-Cys) or carrier (5-FU/PBS) as a control for 52 days. Other control mice were administered with PBS instead of 5-FU (PBS) and were not further treated. Blood was taken regularly and platelet counts were performed. (n=4-8; error bars represent SE; *: $p<0.05$; **:$p\leq0.005$, relative to 5-FU/PBS).**C-E:** Mice were sub-lethally irradiated and injected with VEGFR-3 blocking antibodies, isotype control Ig or carrier (PBS) every other day for 20 days. Blood was taken regularly and platelet counts were performed. (**C**). Bone marrow was isolated 20 days post irradiation, then stained with CD41 specific antibodies and fixed. The DNA of the cells was then stained with Draq5 and the number (**D**) and ploidy (**E**) of CD41 positive cells was subsequently analysed in FACS. (n=8; error bars represent SE; *: $p<0.05$). **F-H:** Mice were sub-lethally irradiated and received daily injections of either VEGF-C-Cys or carrier (PBS) as a control for 20 days. Blood was taken regularly and platelet counts were performed. (**F**). Bone marrow was isolated 20 days after irradiation, stained with CD41 specific antibodies, and then fixed. The DNA of the cells was then stained with Draq5 and the number (**G**) and ploidy (**H**) of CD41 positive cells was subsequently analysed in FACS. (n=8; error bars represent SE; *: $p<0.05$; **:$p<0.01$).