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ORIGINAL ARTICLE

Comparison of endothelial promoter efficiency and specificity in mice reveals a subset of Pdgfb-positive hematopoietic cells

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Essentials

- To reliably study the respective roles of blood and endothelial cells in hemostasis, mouse models with a strong and specific endothelial expression of the Cre recombinase are needed.
- Using *mT/mG* reporter mice and conditional $JAK2^{V617F/WT}$ mice, we compared *Pdgfb-iCreERT2* and *Cdh5(PAC)-CreERT2* with well-characterized *Tie2-Cre* mice.
- Comparison of recombination efficiency and specificity towards blood lineage reveals major differences between endothelial transgenic mice.
- Cre-mediated recombination occurs in a small number of adult hematopoietic stem cells in *Pdgfb-iCreERT2;* $JAK2^{V617F/WT}$ transgenic mice.

Summary. *Background:* The vessel wall, and particularly blood endothelial cells (BECs), are intensively studied to better understand hemostasis and target thrombosis. To understand the specific role of BECs, it is important to have mouse models that allow specific and homogeneous expression of genes of interest in all BEC beds without concomitant expression in blood cells. Inducible *Pdgfb-iCreERT2* and *Cdh5(PAC)-CreERT2* transgenic mice are widely used for BEC targeting. However, issues remain in terms of recombination efficiency and specificity regarding hematopoietic cells. *Objectives:* To determine which mouse model to choose when strong expression of a transgene is required in adult BECs from various organs, without concomitant expression in hematopoietic cells. *Methods:* Using *mT/mG* reporter mice to measure recombination efficiency and conditional $JAK2^{V617F/WT}$ mice to assess specificity regarding hematopoietic cells, we compared *Pdgfb-iCreERT2* and *Cdh5(PAC)-CreERT2* with well-characterized *Tie2-Cre* mice. *Results:* Adult *Cdh5(PAC)-CreERT2* mice are endothelial specific but require a dose of 10 mg of tamoxifen to allow constant Cre expression. *Pdgfb-iCreERT2* mice injected with 5 mg of tamoxifen are appropriate for most endothelial research fields except liver studies, as hepatic sinusoid ECs are not recombined. Surprisingly, 2 months after induction of Cre-mediated recombination, all *Pdgfb-iCreERT2;*$JAK2^{V617F/WT}$ mice developed a myeloproliferative neoplasm that is related to the presence of JAK2V617F in hematopoietic cells, showing for the first time that Cre-mediated recombination occurs in a small number of adult hematopoietic stem cells in *Pdgfb-iCreERT2* transgenic mice. *Conclusion:* This study provides useful guidelines for choosing the best mouse line to study the role of BECs in hemostasis and thrombosis.

Keywords: blood vessels; Cre recombinase; endothelial cells; hematopoietic system; tamoxifen; transgenic mice.

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Introduction

The normal, healthy endothelium is not simply a passive lining but rather an active participant in many body functions, including maintenance of vascular tone, adaption to changes in blood flow, regulation of fluid exchange and trafficking of cellular components between the intra- and extravascular compartments and maintenance of

normal blood fluidity. Under physiological conditions, endothelium maintains a hemostatic balance between both prothrombotic and antithrombotic factors, as it is able to synthesize, store and release vasoconstrictor, vasodilator, anticoagulant, procoagulant and fibrinolytic proteins [1]. However, endothelial cells can also directly interact with blood cells to promote thrombosis [2–4]. To understand the specific and proper role of endothelial cells in these pathophysiologic processes, it is of particular importance to have mouse models that allow a specific and homogeneous expression of genes of interest in the endothelial cell bed of interest without any concomitant expression in blood cells.

Various transgenic lines were created to study endothelial cell properties in mice, thanks to the development of the Cre/Lox system. Briefly, Cre-recombinase regulated by a tissue-specific promoter excises, when in the nucleus, essential loxP-flanked ("floxed") genes via intrachromosomal recombination to generate conditional knockouts. The first models that were generated allowed a constitutive endothelial cell expression of the Cre-recombinase. These include: *VCAM-Cre* [5], *Tie1-Cre* [6], *Tie2-Cre* [7], *Flk1-Cre* [8] and *Cdh5-Cre* [9,10]. These transgenic lines have been very useful for gene deletion studies but it soon appeared that the expression of the Cre was not restricted to endothelial cells, especially in adult mice, as in most of the models, recombination also occurred in hematopoietic cells [6,9,11–13]. Indeed, lineage-tracing studies have demonstrated endothelial origin of embryonic hematopoietic stem cells [14]. On the contrary, in adults, blood cells derive from hematopoietic stem cells that are distinct from endothelial cells and that no longer express endothelial cells markers.

A way to overcome the concomitant expression of the Cre-recombinase in endothelial and hematopoietic cells is thus to use transgenic lines where Cre expression is induced in adult endothelial cells after tamoxifen injection, such as *Tie2-CreERT2* [15], *endothelial-SCL-Cre^{ERT}* [16], *Cdh5-CreERT2* [17], *Cdh5(PAC)-CreERT2* [18] and *Pdgfb-iCreERT2* [19]. However, in these mice, expression of the Cre-recombinase in hematopoietic cells has not been carefully examined.

When designing experiments to study endothelial cell properties, especially in the field of thrombosis or angiogenesis, we should keep in mind that endothelium is heterogeneous, as endothelial cells adapt to their local tissue environment [20]. When choosing among endothelial-specific Cre mouse models, it is thus particularly important to check that endothelial cells express the Cre-recombinase in the organ of interest highly enough to induce an efficient and homogenously distributed recombination.

Our aim was to determine which endothelial mouse models to use when a homogeneous and strong expression of a transgene is required in different vascular territories, with no concomitant expression in hematopoietic cells. To address the question of endothelial expression specificity and homogeneity, we used two complementary approaches: the use of *mT/mG* mice [21] to report homogeneity and the conditional flexed *JAK2$^{V617F/WT}$* mice [22] to report specificity regarding hematopoietic cells, as expression of JAK2V617F in hematopoietic stem cells gives rise to a myeloproliferative disease [23] and could reveal a small subset of recombined hematopoietic stem cells. We compared two inducible transgenic mouse lines, *Pdgfb-iCreERT2* [19] and *Cdh5(PAC)-CreERT2* [18], with the well-characterized constitutive *Tie2-Cre* [7] transgenic mouse line, which allows a strong level of recombination in endothelial cells of all organs but also a high level of recombination in the hematopoietic lineage [12]. We thus decided to use them as a positive control for endothelial expression, knowing it was not an appropriate model for specific endothelial expression. We observed that *Pdgfb-iCreERT2* and *Cdh5(PAC)-CreERT2*-mediated recombination occurs in the vast majority of blood endothelial cells in the brain, retina, lungs, kidney and mesentery, but that *Cdh5(PAC)-CreERT2* required higher doses of tamoxifen than *Pdgfb-iCreERT2*. Using flexed *JAK2 (JAK2$^{V617F/WT}$)* mice [22], we did not detect any recombination in hematopoietic cells in *Cdh5(PAC)-CreERT2* mice. On the contrary, we observed that all *Pdgfb-iCreERT2;JAK2$^{V617F/WT}$* mice developed a JAK2V617F-positive myeloproliferative neoplasm 2 months after tamoxifen delivery, showing for the first time that Cre-mediated recombination occurs in a small number of adult hematopoietic cells in *Pdgfb-iCreERT2* transgenic mice.

Materials and methods

Animals

This study was conducted in accordance with both Bordeaux University Institutional Committee guidelines (committee CEEA50) and those in force in the European Community for experimental animal use (L358-86/609/EEC). Reporter mice (B6.129[Cg]-Gt[ROSA]26Sortm4 [ACTB-tdTomato,- EGFP]Luo/J), hereafter called *mT/mG* mice [21], were purchased from Charles River. Double-heterozygous *Tie2-Cre;mT/mG*, *Cdh5(PAC)-CreERT2;mT/mG* and *Pdgfb-iCreERT2;mT/mG* mice were generated by crossing *mT/mG* mice with *Tie2-Cre* [7], *Cdh5(PAC)-CreERT2* [18] and *Pdgfb-iCreERT2* [19] mice, respectively. We used two sets of controls: tamoxifen-injected–*Cre-negative;mT/mG* mice and vehicle-injected–*Cre-positive;mT/mG* mice. The conditional flexed *JAK2 (JAK2$^{V617F/WT}$)* mice were generously provided by J.L. Villeval [22]. These mice allow expression of wild-type JAK2 when the Cre is not expressed and a constitutively activated form of JAK2, JAK2V617F, after Cre-mediated recombination. The *Cre-negative;JAK2$^{V617F/WT}$* mice were used as controls. All groups used in this work were equally matched for genders.

Tamoxifen administration

To induce Cre activity in adult *Pdgfb-iCreERT2* mice, five intraperitoneal tamoxifen (10 mg mL^{-1} in ethanol/peanut oil) (Sigma, St. Louis, MO, USA) injections of 100 μL (1 mg) were given for 5 consecutive days to 5–6-week-old animals. At this age, mice weighed between 17 and 22 g, so for a mean weight of 20 g, 1 mg tamoxifen is 50 mg kg^{-1}. For *Cdh5(PAC)-CreERT2* mice, we used two different protocols: 1 mg IP for 5 consecutive days (5 mg total, i.e 250 mg kg^{-1}) and 1 mg IP for 5 consecutive days, 2 consecutive weeks (10 mg total, i.e 500 mg kg^{-1} total). The phenotypes of mutant mice were analyzed 1 month after injection, unless otherwise specified.

Microscopy/confocal microscopy

Adult tissues were isolated from anesthetized mice perfused with 4% ParaFormAldehyde (PFA). Retinal whole-mount preparations were dissected after 30 min fixation in 4% PFA and then mounted in Vectashield mounting medium. Mesenteries were washed in phosphate-buffered saline (PBS). Whole mount brains were washed in PBS, fixed in 4% PFA and 50-μm sections were obtained with a Leica Vibratome (Leica Biosystems, Wetzlar, Germany). Retinal, mesenteric and brain vasculatures were imaged with a fluorescent microscope (AxioObserver, Zeiss, Oberkochen, Germany) and analyzed using ZEN imaging software (Zeiss). Livers, hearts, lungs, kidneys, brains and femurs were fixed overnight in 4% PFA, cryoprotected in sucrose, embedded in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek Europe B.V. AV Alphen aan den Rijn, Netherlands) and cryosectioned. Before staining, organ cuts were post-fixed for 30 minutes with 4% PFA. Antibodies used for immunofluorescence staining were rat anti-mouse CD31 (BD Pharmingen, Franklin Lakes, NJ, USA) and goat anti-rat IgG conjugated to Alexa Fluor® 647 (Invitrogen, Carlsbad, CA, USA). All tissues were imaged with a confocal microscope (Olympus FV 1000, Tokyo, Japan) and analyzed by Imaris software (Bitplane, Zurich, Switzerland).

Quantification of Cre-recombination efficiency

The estimation of the efficiency of the Cre-mediated recombination in the endothelial cells in the liver, heart, lung, kidney and brain was performed using "Image J" software by measuring the surface of double-stained structures (EGFP+;CD31+) per field over the surface of the single-stained CD31+ structure (CD31 endothelial cells) per field. The ratio of the "double-stained EGFP+; CD31+ cells over the total CD31+ endothelial cells" was then represented as a percentage.

Blood count and histopathology analysis

Blood counts were determined using an automated counter (scil Vet abc Plus+). Spleens were fixed in 4% neutral buffered formalin and embedded in paraffin. Then 7-μm-thick spleen sections were stained with hematoxylin, eosin and safran and images were taken using a Nikon Microphot-FXA microscope with a ×10 objective, captured with an Axio Cam HR digital camera (Zeiss, Germany), and analyzed using ZEN imaging software (Zeiss).

Isolation of primary endothelial cells from mouse tissues

We used kidney to isolate endothelial cells, as this protocol was routinely used in our laboratory, with a good output in terms of efficiency and purity [24]. Kidneys were removed, minced in slices and endothelial cells were isolated after magnetic immunoseparation with anti-CD31 (Pharmingen) and anti-endoglin (Santa Cruz) [24].

Cell sorting and flow cytometry analysis

For cell sorting, primary kidney endothelial cells were stained with CD-31 FITC (Becton Dickinson, Franklin Lakes, NJ, USA). Erythroid and granulocyte bone marrow cells were, respectively, stained with Ter119 PE and Gr1 APC antibodies (Becton Dickinson). Cells were isolated using a FACS Aria III cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). For flow cytometry analysis in Cre;mT/mG mice, bone marrow cells were stained with TER-119 APC and Gr-1 APC (Becton Dickinson) and analyzed on an Accuri C6 flow cytometer (BD Biosciences). Data were interpreted using BD Accuri C6 Analysis Software.

DNA purification and quantitative allele-specific PCR

Genomic DNAs were purified using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany). For quantification of wild-type (WT) and mutated JAK2 DNA, quantitative allele specific PCR from gDNA was conducted using the GAAGCAGCAAGCATGATGAG forward primer and the AAAGACAAGCAGCTGGCTT TACTTAC reversed primer for PCR amplification and the GTGTCTGTTTCTGTGGA $JAK2^{V617F}$ probe or the GTGTCTGTGTCTGTGGA $JAK2^{WT}$ probe to identify amplified fragments from the mutated or WT JAK2 DNA, respectively. It was performed using a 7500 Real Time PCR System AB (Applied Biosystems, Foster City, CA, USA) and analyzed with associated software.

Statistical analysis

Results were expressed as mean ± SEM and data were analyzed with the two-way ANOVA test followed by the

"Bonferroni" post-test using Graphpad PRISM software. A value of $P \leq 0.05$ was considered to be statistically significant.

Results

Endothelial expression of the Cre-recombinase is efficient in Tie2-Cre *and* Pdgfb-iCreERT2 *mice*

To assess the efficiency of Cre expression, we used double-fluorescent mT/mG reporter mice, which allow the direct live visualization and distinction of the occurrence of the Cre-mediated recombination in a spatial and temporal manner [21]: when the Cre is not expressed, cells are Tomato positive and when the Cre is expressed, cells are no longer Tomato but EGFP positive. We first performed whole mount vessel analysis in the retina, mesentery and brain (Fig. 1) and then looked more closely at blood endothelial cells using CD31 staining and confocal examination in various organs of interest: the liver, heart, lungs, kidney and brain (Figs. 2 and 3). As expected, in 10-week-old *Tie2-Cre;mT/mG* mice, all vessel networks and 77.4 ± 11.7% of CD31+ cells were EGFP positive and Tomato negative in all tissues examined, indicating an efficient Cre-mediated recombination (Figs. 1–3, Fig. S1A). We then characterized inducible *Pdgfb-iCreERT2* mice 5 weeks after five consecutive intraperitoneal injections of 1 mg tamoxifen, a dose commonly used in the literature [19,25]. We used two sets of controls: tamoxifen-injected *Cre-negative;mT/mG* mice and vehicle-injected *Cre-positive;mT/mG* mice, the latter allowing us to investigate whether we could detect a basal expression of the Cre recombinase in the absence of tamoxifen. We did not detect any EGFP expression in tamoxifen-injected *Cre-negative;mT/mG* mice (data not shown). Analysis of the vascular network in vehicle-injected *Pdgfb-iCreERT2;mT/mG* mice revealed a weak EGFP expression in small portions of retinal vessels and in rare CD31-positive cells from the brain and heart (Fig. S2). Because *Pdgfb-iCreERT2* mice contain a sequence coding for EGFP downstream of iCreERT2, we wondered whether the EGFP staining we detected was a result of the presence of EGFP in the construct or endogenous Cre expression. Analysis of Tomato expression revealed that EGFP-positive cells were also Tomato positive, thus ruling out the hypothesis of endogenous Cre expression. We then analyzed tamoxifen-injected *Pdgfb-iCreERT2;mT/mG* mice and noted a high efficiency of recombination in all vascular networks and CD31+ cells of all tissues (78.8 ± 15.9%) except the liver (26 ± 11%) (representative images in Figs. 1 and 2 and Fig. S1B, and details of quantification per organ in Fig. 3). Indeed, in the liver we did not detect any Cre expression in the sinusoidal endothelial cells, whereas the ones from the centrilobular vein were efficiently recombined (Fig. 2 and Fig. S1B).

Efficient blood endothelial expression of the Cre-recombinase in Cdh5(PAC)-CreERT2 *mice requires higher doses of tamoxifen than* Pdgfb-iCreERT2 *mice*

Cdh5(PAC)-CreERT2 mice are intensively used both for adult and developmental studies, with total doses of tamoxifen that vary between 3 mg IP [26,27] and 15 mg IP [28] in 4–6-week-old mice. We thus decided to analyze recombination efficiency in a first set of 5–6-week-old mice, which we injected with 5 mg tamoxifen IP (250 mg kg^{-1}), the same dose that we had used for *Pdgfb-iCreERT2* mice. As for *Pdgfb-iCreERT2* mice, we used vehicle-injected *Cdh5(PAC)-CreERT2;mT/mG* mice to look for any basal expression of the Cre. We could not detect any EGFP expression in the retina, liver, heart, lungs, kidney and brain of both types of control mice (data not shown). In *Cdh5(PAC)-CreERT2;mT/mG* mice ($n = 18$) 5 weeks after five consecutive IP injections of 1 mg tamoxifen, we surprisingly observed that efficiency of Cre-mediated recombination was highly variable (Figs. 1–3, Fig. S1C,D), with percentages of EGFP+ CD31+ cells ranging between 10 and 95% depending on the mice. Variable recombination efficiency occurred within littermates, injected on the same 5 consecutive days with the same batch of tamoxifen. We then decided to increase the total dose of tamoxifen to 10 mg (1 mg for 10 days IP; i.e. 500 mg kg^{-1}) We analyzed a total of 11 mice and observed a complete recombination in blood vessels from the retina, liver, heart, lungs, kidney and brain (Figs. 1–3). These results thus demonstrate *Cdh5(PAC)–CreERT2* mice need 10 mg of tamoxifen for constant recombination in all mice.

The Cre-recombinase is expressed in adult hematopoietic cells in Tie2-Cre;mT/mG *transgenic mice, but not in* Pdgfb-iCreERT2;mT/mG *and* Cdh5(PAC)–CreERT2;mT/mG *mice*

Because we aimed to determine which mouse model is blood endothelial specific, without any Cre expression in blood cells, we then investigated whether Cre-mediated recombination also occurs in adult hematopoietic cells. To analyze the efficiency of Cre-mediated recombination in megakaryocytes, we observed femur cryo-sections by confocal microscopy. As expected, all megakaryocytes and other hematopoietic cells were EGFP+ in adult constitutive *Tie2-Cre;mT/mG* mice (Fig. 4A, arrowheads). In inducible *Pdgfb-iCreERT2;mT/mG* mice injected with 5 mg tamoxifen and *Cdh5(PAC)-CreERT2;mT/mG* mice injected with 10 mg tamoxifen, 1 and 2 months after tamoxifen injection, all hematopoietic cells, including megakaryocytes (Fig. 4A, empty arrowheads) expressed tdTomato, suggesting that no hematopoietic expression of the Cre had occurred. Bone marrow capillaries appeared in green (Fig. 4A, arrows) in accordance with Cre-mediated recombination in bone marrow endothelial cells. To confirm these results and quantitatively analyze Cre

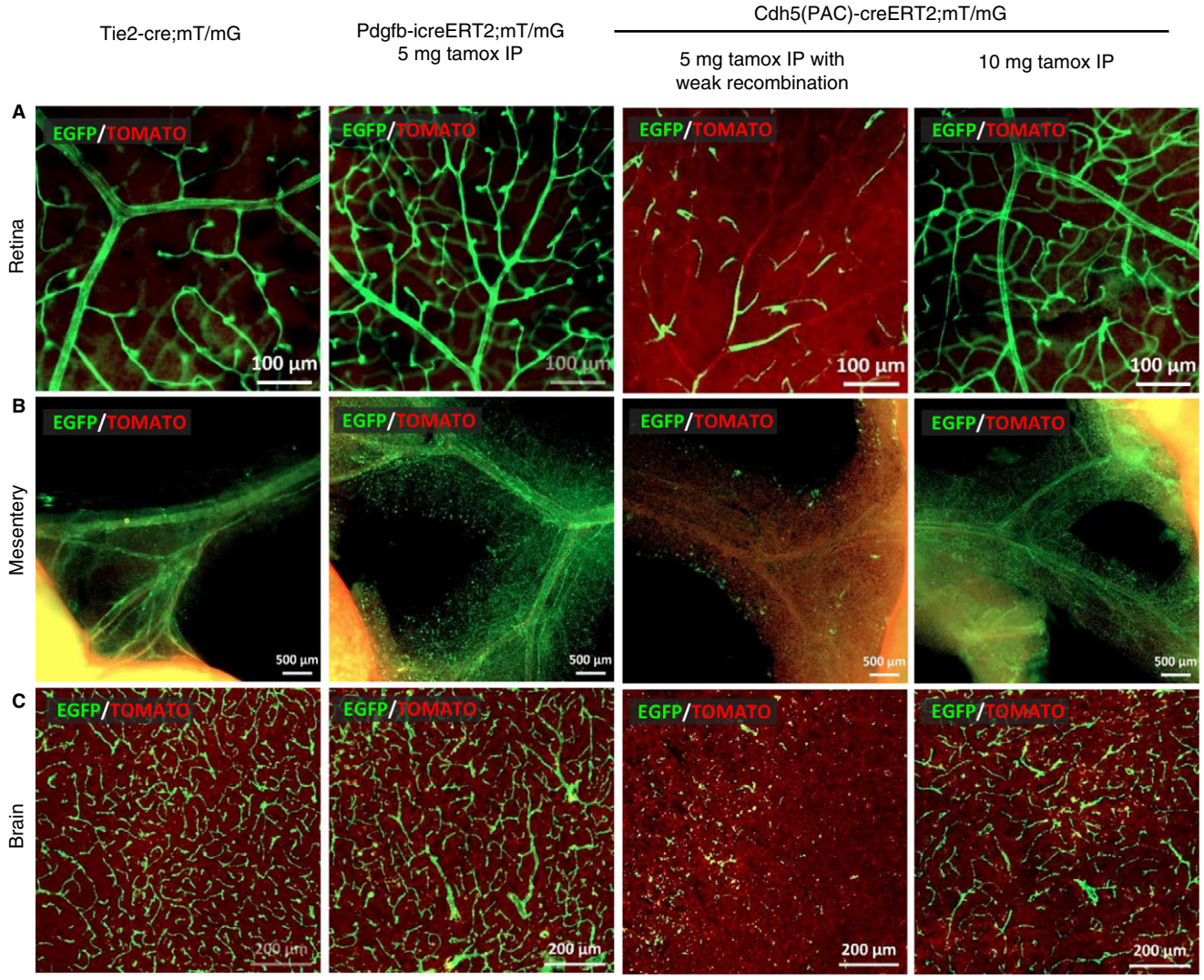


Fig. 1. The Cre-recombinase is efficiently expressed in the vascular network of adult retina, mesenteric territory and brain in *Tie2-Cre*, *Pdgfb-iCreERT2* mice receiving 5 mg tamoxifen and *Cdh5(PAC)–CreERT2* mice injected with 10 mg tamoxifen. *mT/mG* mice that constitutively express the tandem dimer Tomato protein (tdTomato) "mT" red were crossed with endothelial promoter specific-Cre mice. In the resulting progeny, cells where the endothelial promoter is expressed will express the Cre and thus the enhanced green fluorescent protein (EGFP) "mG" green instead of the red tdTomato. Mice were analyzed at 10 weeks of age. Fluorescence imaging of the vasculature of the adult retina (A), mesentery (B) and brain thick sections (C) in constitutive *Tie2-Cre;mT/mG* mice, inducible *Pdgfb-iCreERT2;mT/mG* and *Cdh5(PAC)-CreERT2;mT/mG* mice 5 weeks after tamoxifen injection. Representative images of constitutive *Tie2-Cre;mT/mG*, inducible *Pdgfb-iCreERT2;mT/mG* mice injected with 5 mg tamoxifen, and *Cdh5(PAC)-CreERT2;mT/mG* mice injected with 5 mg or 10 mg IP tamoxifen are shown. For inducible *Cdh5(PAC)-CreERT2;mT/mG* mice, variable recombination efficiency was observed, with 12/18 mice displaying an efficient cre-mediated recombination in almost all vessels, whereas the six other mice showed a weak recombination in all analyzed tissues (as shown in this figure).

expression in erythroid (Ter119) and granulocytic (Gr1) cells, bone marrow was analyzed by flow cytometry. We observed that 99% of Ter119+ and Gr1+ cells were EGFP+ in constitutive *Tie2-Cre;mT/mG* mice (Fig. 4B and C). This result was not surprising as Tang *et al.* already reported Cre-mediated recombination in 82% of hematopoietic cells in Tie2-Cre;Rosa26R-EYFP mice [12]. By contrast, we did not observe any EGFP+ hematopoietic cells in inducible *Cdh5(PAC)-CreERT2;mT/mG* and *Pdgfb-iCreERT2;mT/mG* mice (Fig. 4B and C). Altogether the results obtained with *mT/mG* reporter mice suggest, as previously published, that Cre is not expressed in the hematopoietic lineage of adult inducible *Cdh5(PAC)-CreERT2* and *Pdgfb-iCreERT2* mice.

Pdgfb-iCreERT2;JAK2$^{V617F/WT}$ *mice develop a late myeloproliferative disease 10 weeks after induction of JAKV617F expression*

To further address the question of concomitant Cre-mediated recombination in hematopoietic cells, we crossed endothelial-Cre mice with conditional *JAK2*$^{V617F/WT}$ mice [22]. These mice allow expression of wild-type JAK2 when the Cre is not expressed and a constitutively activated form

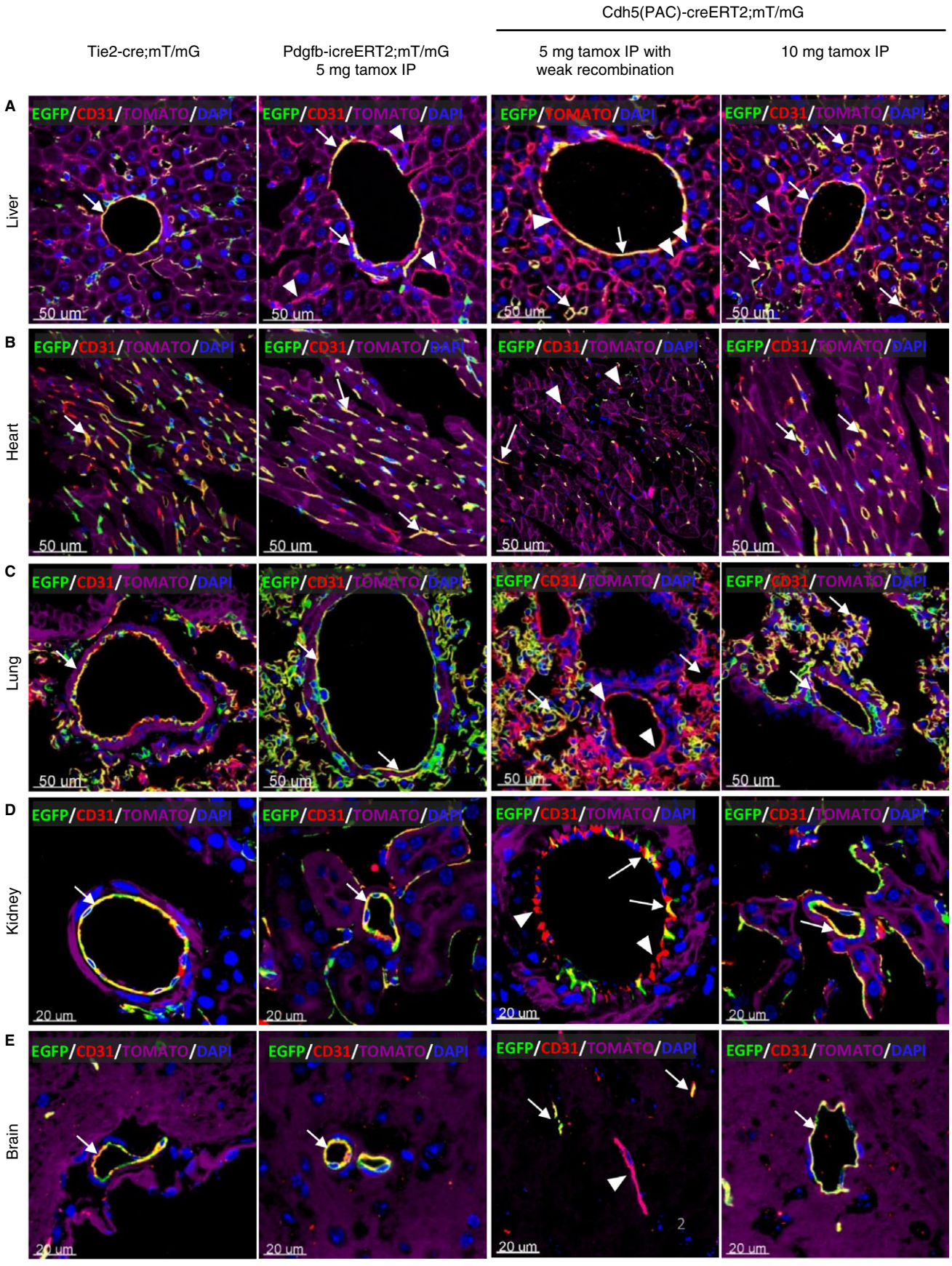


Fig. 2. Recombination in endothelial cells from the liver, heart, lung, kidney and brain is almost complete in *Tie2-Cre*, *Pdgfb-iCreERT2* mice and *Cdh5(PAC)–CreERT2* mice injected with 10 mg tamoxifen IP. (A–E) Confocal imaging of endothelial cells in the liver (A), heart (B), lung (C), kidney (D) and brain (E). Endothelial cells are labelled with anti-CD31 (red), and co-localization of CD31 and enhanced green fluorescent protein (EGFP) appears yellow (arrows). Non-recombined endothelial cells (EGFP negative) labelled with CD31 (red) are indicated by an arrowhead. As expected, images of constitutive *Tie2-Cre;mT/mG* mice show an efficient recombination in almost all blood endothelial cells. Efficient recombination was observed in all *Pdgfb-iCreERT2;mT*/mG. Note the absence of recombination in endothelial cells of the sinusoid blood vessels of the liver in *Pdgfb-iCreERT2;mT/mG* mice (arrowheads). For *Cdh5(PAC)-CreERT2;mT/mG* mice, the same well-recombined 12/18 mice shown in Fig. 1 also demonstrated an efficient recombination in all analyzed tissues. Consistent with the results in Fig. 1, a weak EGFP expression was observed in the other six mice (as shown here). For all images: Nucleus, DAPI, blue; EGFP, green. For (A–E) CD31 is red and tdTomato is purple.

of JAK2, JAK2V617F, after Cre-mediated recombination. Because the expression of JAK2V617F in hematopoietic stem cells gives rise to a myeloproliferative neoplasm [23], we reasoned that expression of the Cre-recombinase in a small subset of hematopoietic stem cells, even if not detectable using mT/mG system, would become visible using $JAK2^{V617F/WT}$ mice. We thus measured hematocrit, platelet and granulocyte count in inducible *Pdgfb-iCreERT2* mice injected with 5 mg tamoxifen IP and *Cdh5(PAC)-CreERT2* mice injected with 10 mg tamoxifen IP, up to 24 weeks after injection. We used constitutive *Tie2-Cre* mice as positive controls. As reported in the literature [29], we observed that *Tie2-Cre;*$JAK2^{V617F/WT}$ mice developed severe thrombocytosis and leukocytosis (Fig. 5A). All blood counts remained normal in the 15 *Cdh5(PAC)-CreERT2;*$JAK2^{V617F/WT}$ mice we analyzed (Fig. 5B). By contrast, in *Pdgfb-iCreERT2;*$JAK2^{V617F/WT}$ mice we noted, 6 weeks after tamoxifen induction, a slight increase in hematocrit (Fig. 5B). Twelve weeks after Cre-mediated recombination induction, all blood cell counts were markedly increased, suggesting that these mice had developed a myeloproliferative neoplasm (Fig. 5B). Because spleen size and architecture are known to be altered in mice with myeloproliferative neoplasms, we analyzed spleen features from 10-week-old *Tie2-Cre;*$JAK2^{V617F/WT}$ mice and *Pdgfb-iCreERT2;*$JAK2^{V617F/WT}$ mice, 5 and 10 weeks after tamoxifen injection. As expected, *Tie2-Cre;*$JAK2^{V617F/WT}$ mice had splenomegaly with a 9.6-fold increase in spleen weight (Fig. 5C,D). *Pdgfb-iCreERT2;*$JAK2^{V617F/WT}$ mice had normal spleen size and weight 5 weeks after tamoxifen induction, but exhibited splenomegaly 10 weeks after (Fig. 5C,D). Histological analysis of the spleen revealed red pulp hyperplasia and clustered megakaryocytes with a multilobulated nucleus in *Tie2-Cre;*$JAK2^{V617F/WT}$ and *Pdgfb-iCreERT2;*$JAK2^{V617F/WT}$ mice, when analyzed 10 weeks after tamoxifen induction (Fig. 5E). These features resemble the ones observed when JAK2V617F is expressed in hematopoietic cells [22].

The myeloproliferative disease is a result of hematopoietic expression of JAK2V617F, showing that Cre-mediated recombination occurs in a small number of adult hematopoietic stem cells in Pdgfb-iCreERT2;$JAK2^{V617F/WT}$ *mice*

Two mechanisms were possible to explain why *Pdgfb-iCreERT2;*$JAK2^{V617F/WT}$ mice exhibit a late myeloproliferative phenotype 10 weeks after tamoxifen induction. The first hypothesis was that Cre-mediated recombination had occurred in only a small number of hematopoietic stem cells. Because expression of JAK2V617F in mouse early hematopoietic cells gives them a strong competitive advantage [22], the blood phenotype, not visible at first, would become apparent after JAK2V617F progenitors have had enough time to expand. The second hypothesis was that JAK2V617F expression was strictly restricted to blood endothelial cells, but that JAK2V617F blood endothelial cells could promote hematopoietic stem cell proliferation [30], thus giving rise to a myeloproliferative disease with JAK2 wild-type blood cells. To answer this question, we sorted bone marrow erythroid and granulocyte cells and measured JAK2V617F allele burden. As expected, almost all hematopoietic cells were JAK2V617F+ in *Tie2-Cre;*$JAK2^{V617F/WT}$ mice (Table 1). Very interestingly, in 10 weeks post-tamoxifen *Pdgfb-iCre ERT2;*$JAK2^{V617F/WT}$ mice, almost all hematopoietic cells also carried the JAK2V617F mutation. Remarkably, we could also detect a small number of JAK2V617F hematopoietic cells in *Pdgfb-iCreERT2;*$JAK2^{V617F/WT}$ mice, 5 weeks after tamoxifen injection, when blood parameters were still within the normal range (Table 1). As expected, we confirmed that kidney CD31+ endothelial cells isolated from *Tie2-Cre;*$JAK2^{V617F/WT}$ and *Pdgfb-iCreERT2;*$JAK2^{V617F/WT}$ mice were JAK2V617F positive at all time-points (Table 1). Overall, these results provide evidence that the myeloproliferative phenotype observed in *Pdgfb-iCreERT2;*$JAK2^{V617F/WT}$ mice originates from endogenous expression of JAK2V617F in some rare hematopoietic stem cells, thus demonstrating that *Pdgfb-iCreERT2* mice are not strictly endothelial specific.

Discussion

To decipher the specific role of blood endothelial cells in the pathogenesis of thrombosis in mice, mouse models that allow an efficient and specific targeting of endothelial cells are needed. The endothelial-specific targeting is particularly important with regard to blood cells, as platelets, leukocytes and red cells are known to interact with the endothelium, thus promoting thrombosis. These last 15 years many groups used the Cre/Lox system to develop transgenic mouse lines with the objective to target Cre expression to the endothelial cell compartment.

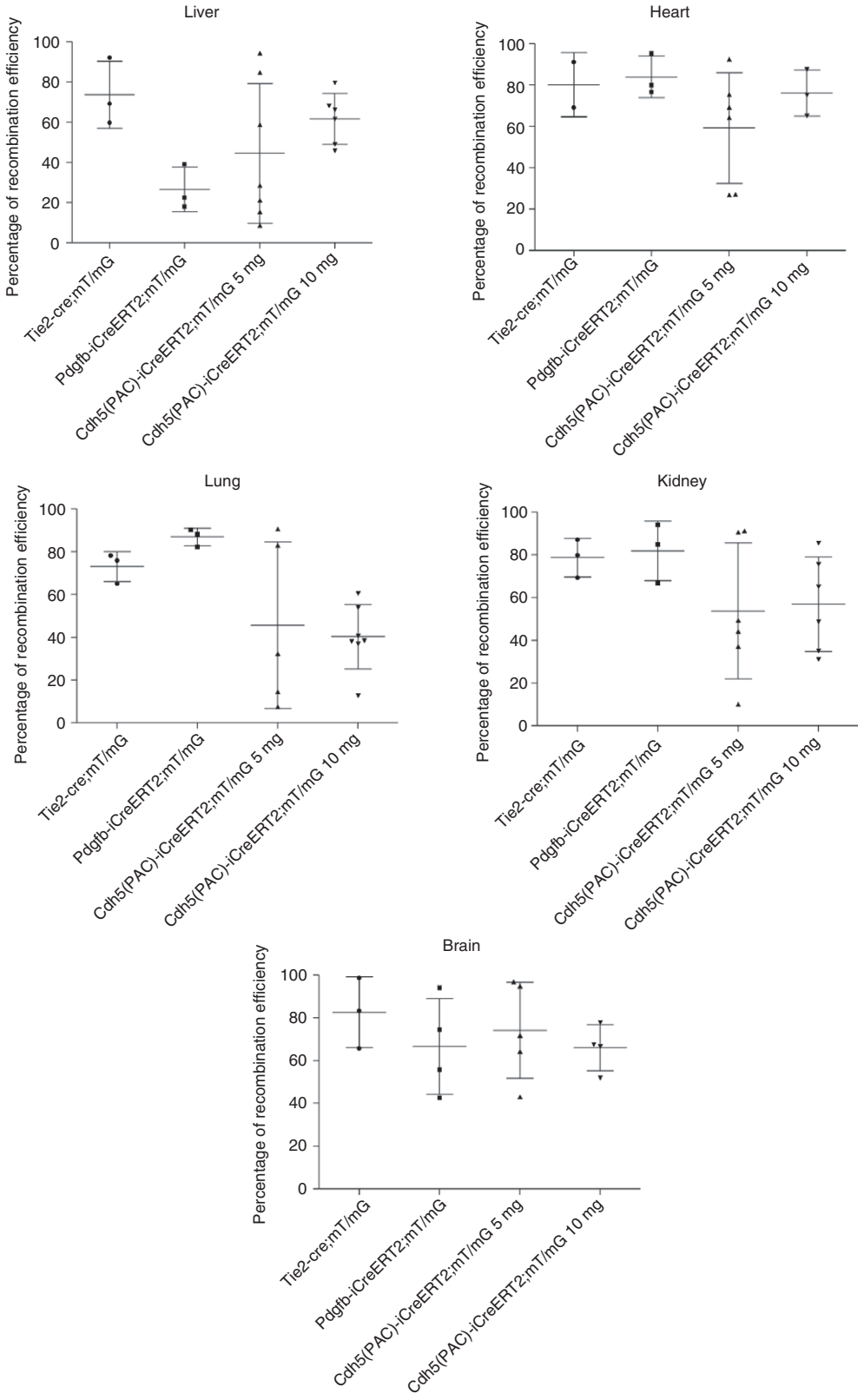


Fig. 3. Quantification of recombination efficiency in the liver, heart, lung, kidney and brain in *Tie2-Cre*, *Pdgfb-iCreERT2* and *Cdh5(PAC)–CreERT2 mice*. Analysis of Cre-mediated recombination efficiency in endothelial cells of rich-vascular tissues was estimated as the percentage of double-stained enhanced green fluorescent protein (EGFP)/CD31+ cells over total CD31+ endothelial cells. For the quantification, the efficiency of the Cre-mediated recombination in the endothelial cells was estimated in at least three mice. Results are presented as mean value ± SEM.

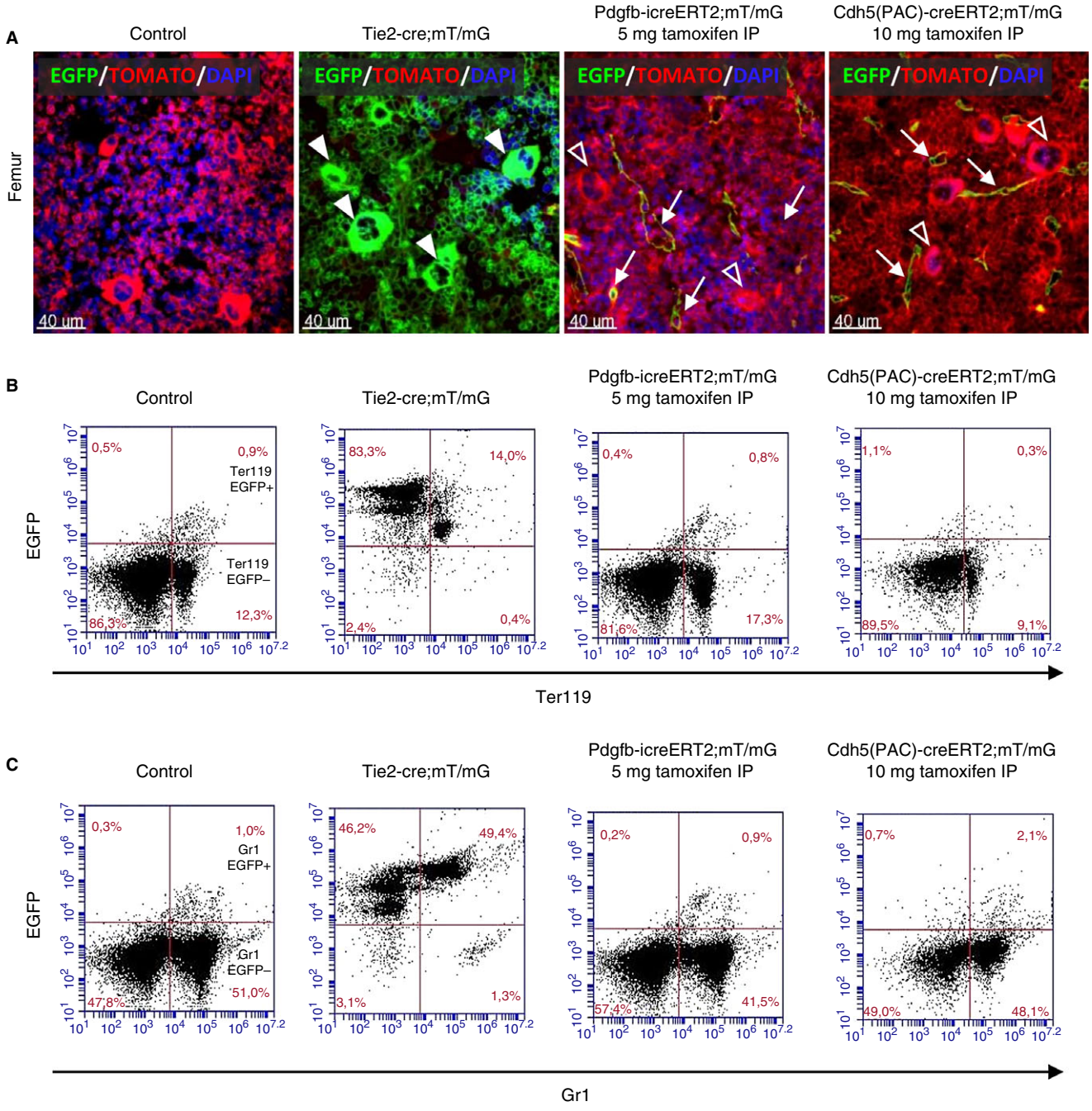
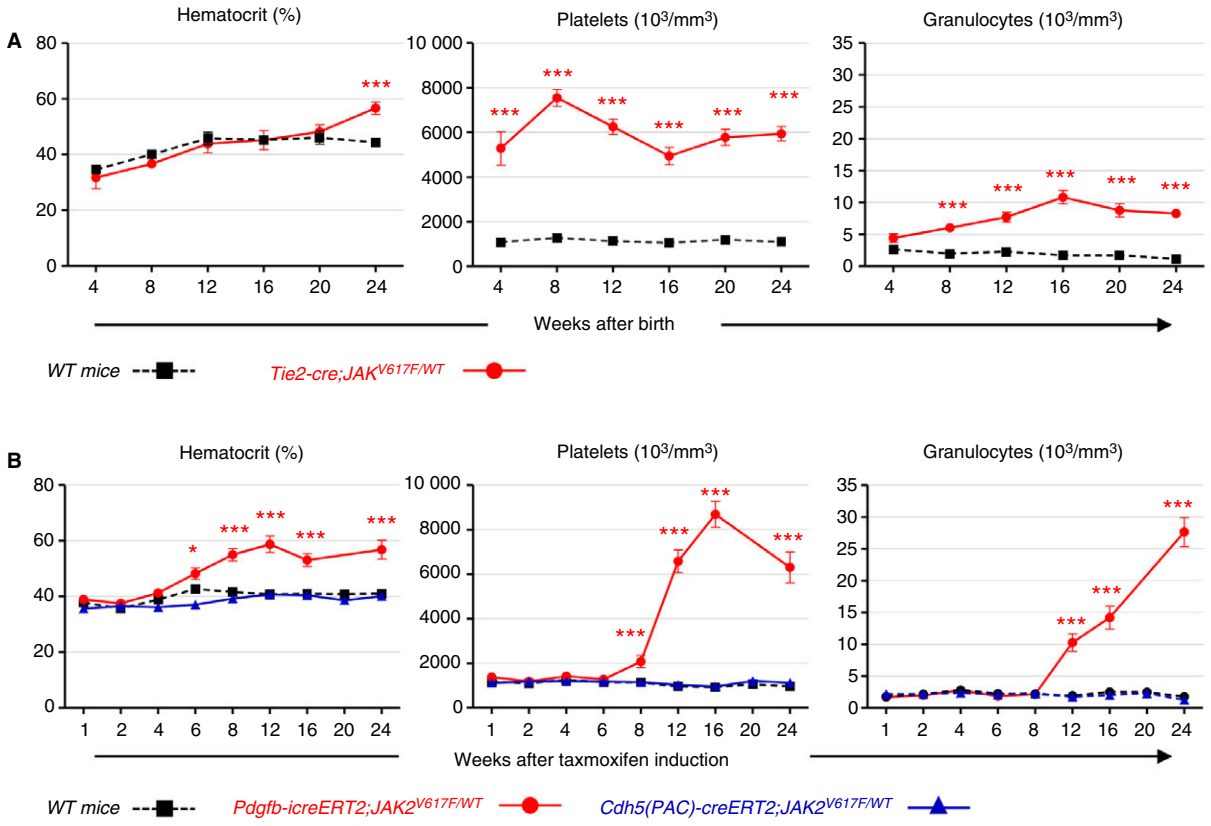
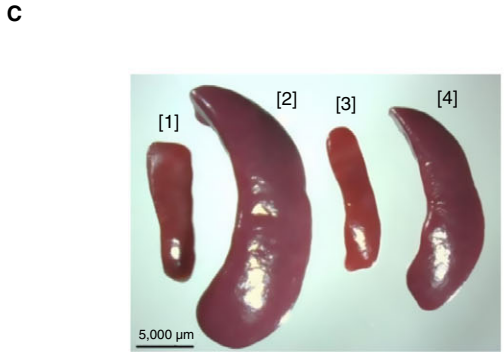
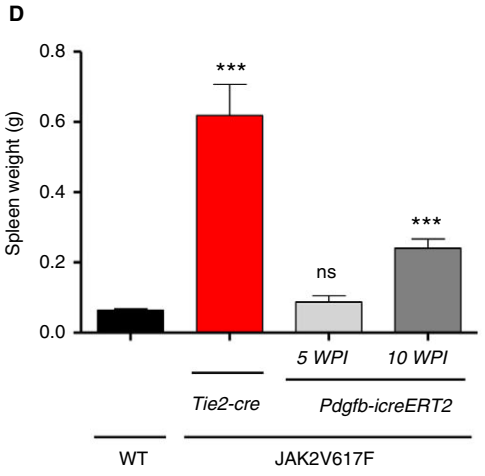


Fig. 4. The Cre-recombinase is expressed in hematopoietic cells in *Tie2-Cre;mT/mG* transgenic mice, but not in *Pdgfb-iCreERT2;mT/mG* and *Cdh5(PAC)–CreERT2;mT/mG* mice 5 and 10 weeks after tamoxifen injection. (A) Confocal imaging of hematopoietic cells in adult femur sections of constitutive *Tie2-Cre;mT/mG* mice shows, as expected, that almost all megakaryocytes, identified by their size (arrowheads), and all hematopoietic cells are enhanced green fluorescent protein (EGFP)+ (green). In inducible *Cdh5(PAC)-CreERT2;mT/mG* and *Pdgfb-iCreERT2;mT/mG* mice all hematopoietic cells are red, including megakaryocytes (empty arrowheads). Recombined vessels appear in green (arrows), showing that Cre is efficiently expressed in marrow sinusoid endothelial cells. Nucleus, DAPI, blue; EGFP, green; tdTomato, red. Images are representative of at least three mice. (B, C) Flow cytometry analysis of hematopoietic cells isolated from femur bone marrow shows that almost all erythroid Ter119-positive (B) and granulocyte Gr1-positive (C) cells are EGFP+ in constitutive *Tie2-Cre;mT/mG* mice compared to control mice. In inducible *Cdh5(PAC)-CreERT2;mT/mG* (10 mg tamoxifen) and *Pdgfb-iCreERT2;mT/mG* mice (5 mg tamoxifen) almost all erythroid and granulocyte cells are EGFP-. *Cre-negative;mT/mG-positive* mice, which express ubiquitously the tdTomato red protein, were used as negative controls. The dot plots are representative of at least five mice. The proportion of gated events is indicated.

To our knowledge, there are now at least six mouse strains that allow a constitutive [5–10] expression of a transgene in blood endothelial cells and five mouse strains after tamoxifen administration [15–19]. The issue of specificity, and hence reliability, when using Cre mice is a major concern. For example, recent studies in the broadly







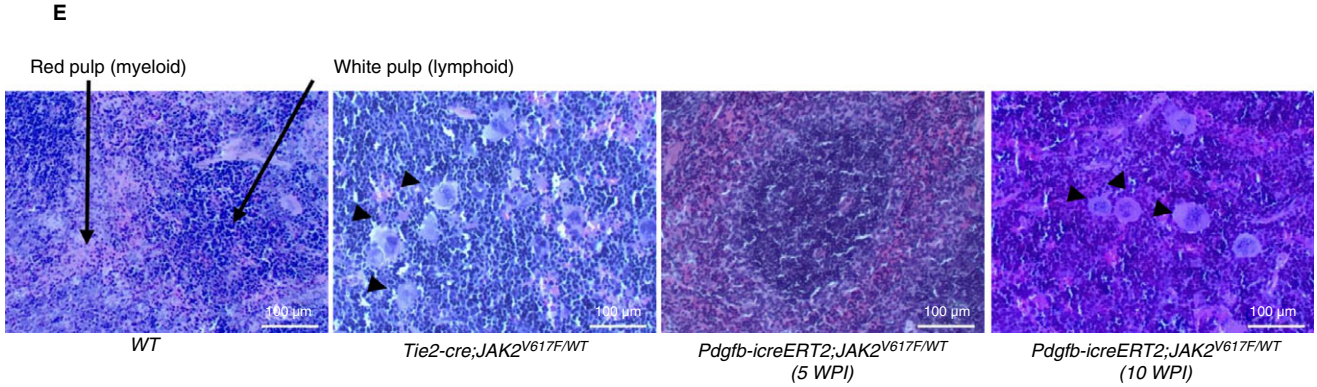


Fig. 5. *Pdgfb-iCre^{ERT2};JAK2$^{V617F/WT}$* mice develop a myeloproliferative disease 10 weeks after induction of JAK2V617F expression. Conditional flexed *JAK2 (JAK2$^{V617F/WT}$)* mice were crossed with *Tie2-Cre, Cdh5(PAC)-CreERT2* and *Pdgfb-iCreERT2* to allow the expression of JAK2V617F in specific tissues. (A, B) Blood cell parameters were studied until 24 weeks of age in constitutive *Tie2-Cre;JAK2$^{V617F/WT}$* mice (A) and until 24 weeks after tamoxifen induction in inducible *Cdh5(PAC)-CreERT2;JAK2$^{V617F/WT}$* and *Pdgfb-iCreERT2;JAK2$^{V617F/WT}$* mice (B). Constitutive *Tie2-Cre;JAK2$^{V617F/WT}$* mice have a typical blood count of myeloproliferative disorders with a severe thrombocytosis and leukocytosis. *Cdh5(PAC)-CreERT2;JAK2$^{V617F/WT}$* mice keep a normal blood count until 24 weeks after tamoxifen induction. By contrast, *Pdgfb-iCreERT2;JAK2$^{V617F/WT}$* have a normal blood count until 4 weeks after induction but develop an myeloproliferative neoplasms phenotype 6–8 weeks after induction. At least five mice were studied at each time-point. Results in (A, B) are mean value ± SEM, $*P \leq 0.05$, $***P < 0.001$ with the two-tailed unpaired Student's *t*-test compared to the wild-type mice at each time-point. (C, D) Spleen features from 10-week-old *Tie2-Cre;JAK2$^{V617F/WT}$* mice and *Pdgfb-iCreERT2;JAK2$^{V617F/WT}$* mice, 5 and 10 weeks after tamoxifen induction (WPI: weeks post-induction). Spleens collected from constitutive *Tie2-Cre;JAK2$^{V617F/WT}$* mice at 10 weeks old [2] show splenomegaly, with a significant increase in spleen weight compared with controls [1]; inducible *Pdgfb-iCreERT2;JAK2$^{V617F/WT}$* mice keep a normal spleen size and weight 5 weeks after tamoxifen induction [3], but develop splenomegaly and increased spleen weight 10 weeks after tamoxifen induction [4]. Results in (D) are mean value ± SEM, $*P \leq 0.05$, $***P < 0.001$ with the two-tailed unpaired Student's *t*-test compared to the wild-type mice. Results are representative of at least five mice. (E) Histological analysis of the spleen using hematoxylin-eosin-safran (HES) coloration revealed red pulp hyperplasia with a completely blended white pulp and clustered megakaryocytes with multilobulated nucleus (arrowhead) in constitutive *Tie2-Cre;JAK2$^{V617F/WT}$* and inducible *Pdgfb-iCreERT2;JAK2$^{V617F/WT}$* mice 10 weeks after tamoxifen induction. Images are representative of at least five mice.

Table 1 In 10 weeks post-tamoxifen Pdgfb-iCreERT2;JAK2V617F/WT mice, JAK2V617F is expressed in hematopoietic cells

		JAK2V617F+ cells		
Mouse models	Age/time after induction	Ter 119+	Gr1+	CD31+
Negative control	10 weeks	–	–	–
Tie2-cre;JAK2$^{V617F/WT}$	10 weeks	++++	+++	+++
Pdgfb-icreERT2;JAK2$^{V617F/WT}$	5 weeks PI	+	+	+++
	10 weeks PI	+++	+++	++++

Analysis of JAK2V617F allele burden in sorted erythroid and granulocyte cells from *Tie2-Cre;JAK2V617F/WT* and *Pdgfb-iCreERT2;JAK2V617F/WT* mice shows that JAK2V617F mutation is strongly expressed in hematopoietic cells from *Tie2-Cre;JAK2V617F/WT* mice and *Pdgfb-iCreERT2;JAK2V617F/WT* mice 10 weeks after tamoxifen induction (PI: post induction). Note that a slight fraction of hematopoietic cells is JAK2V617F+ in *Pdgfb-iCreERT2;JAK2V617F/WT* mice 5 weeks after tamoxifen induction. Sorted CD31+ endothelial cells isolated from *Tie2-Cre;JAK2V617F/WT* and *Pdgfb-iCreERT2;JAK2V617F/WT* mice kidney are, as expected, JAK2V617F positive. Results are representative of at least three mice and JAK2 Taqman allele specific amplification were performed in duplicate for each sample.

used platelet factor 4 Cre mice, initially described as specific for megakaryocytic restricted recombination [31], revealed recombination in adult hematopoietic progenitor and stem cells [32], and more recently, distal intestinal epithelial cells [33].

To address the question of endothelial expression specificity and homogeneity in various organs, we used *mT/mG Cre* reporter mice [21]. In most of the mouse lines previously described, characterization of the spatial and temporal pattern of Cre expression was achieved using a Cre reporter transgene in which LacZ is expressed only after Cre-mediated recombination [34]. The main problem using this system is that there is no reporter gene expression until Cre excision. Therefore, in tissues without reporter gene expression, Cre activity, even if present, cannot be observed. Besides, contrary to βgal-expressing reporter mice, which require the addition of an exogenous enzymatic substrate for labelling, *mT/mG Cre* reporter mice offer the advantage of being compatible with multicolor microscopy and flow cytometry, without any specific staining.

Using mT/mG reporter mice, we observed that *Pdgfb-iCreERT2* [19] mice allowed a strong expression of the Cre in adult brain blood vessels. This result was surprising, as Claxton *et al.*, in their initial publication using βgal reporter mice, could not detect any recombination in the adult brain. This discrepancy could be explained by a difference in the construction of βgal and mT/mG reporter lines: although both lines have the reporter gene inserted in the ROSA26 locus, mT/mG mice have in addition the strong and ubiquitous CMV β-actin promoter (pCA) driving the expression of the mT/mG cassette. This promoter thus allows a strong expression of mT before Cre excision and strong expression of mG after. It is possible that, in the brain, the ROSA26 locus is not efficient enough to drive the expression of the reporter gene (i.e βgal in *ROSA-lacZ* reporter mice), whereas the addition of the pCA promoter allows it. In this same work, Claxton *et al.* showed only a small number of labelled cells in the liver. Interestingly, in our work, we observed a high efficiency of recombination in endothelial cells from the centrolobular veins, but could not detect any recombination in endothelial cells from sinusoid liver capillaries. We thus want to raise a note of caution while using *Pdgfb-iCreERT2* mice for experiments in liver vessels.

This is of particular importance for researchers working in the field of factor VIII production [35].

We have assessed the recombination efficiency in *Cdh5(PAC)-CreERT2* transgenic mice by counting reporter-positive cells after intraperitoneal injection of tamoxifen. We found that *Cdh5(PAC)-CreERT2* mice needed higher doses and a longer time period of tamoxifen treatment than *Pdgfb-iCreERT2* mice for maximal recombination in blood endothelial cells. This difference in efficacy may be related to the chromatin structure and/or epigenetic regulation of promoter activities [36]. Besides, the need for high doses of tamoxifen suggests that these mice should be carefully monitored, as high doses of tamoxifen can induce toxicity in gastric cells [37] and neurons [38].

Pdgfb is known to be expressed in endothelial cells and megakaryocytes [39]. As expected, using *Pdgfb-iCreERT2* X ROSA-lacZ mice, Claxton *et al*. reported recombination activity in megakaryocytes within 2 days after tamoxifen injection [19]. Mouse megakaryocytes have a definite lifespan (around 4–5 days) and cannot self-renew. It is thus expected that *Pdgfb-iCreERT2* mice, after injection of tamoxifen, will bear Cre recombination in megakaryocytes, leading to production of recombined platelets for a few days after tamoxifen injection, but this megakaryocyte recombination will not last after tamoxifen has been metabolized and megakaryocytes have died. Our experiments with *Pdgfb-iCreERT2;mT/mG* mice indeed confirm this point as we did not observe any EGFP-positive megakaryocytes 1 and 2 months after tamoxifen injection. The observation of recombined hematopoietic cells in *Pdgfb-iCreERT2* mice more than 1 month after injection has, to our knowledge, never been reported. In this work, we revealed a late hematopoietic expression of the transgene only when the latter was JAK2V617F, suggesting that hematopoietic recombination is a rare event that needs an amplification of the hematopoietic compartment to become visible. Two scenarios can account for the presence of recombined hematopoietic cells. (i) A small number of hematopoietic stem cells endogenously express Pdgfb and become recombined after tamoxifen injection simultaneously with endothelial cells. Because the number of Cre expressing (and therefore $JAK2^{V617F/WT}$) hematopoietic stem cells is small, it takes 2 months to be able to detect the expansion of the mutated, proliferative clone. This is in accordance with results from Hasan *et al*. showing that engraftment of 30 hematopoietic stem cells (SLAM cells) is sufficient to generate a myeloproliferative disease with complete penetrance within 2 months delay [22]. (ii) Among adult blood endothelial cells expressing Pdgfb, some are capable of giving rise to adult hematopoietic stem cells, suggesting that endothelium could be hemogenic in adults, as it is in the embryo [40]. Further studies are now needed to decipher which hypothesis is true.

Our study has some limitations. We did not address whether lymphatic endothelial cells are efficiently recombined in endothelial mouse models. Also, we did not test giving the same 5-mg dose but with oral gavage, which could have achieved more rapid and robust recombination [41]. We tried the dose of 4 mg per day for 4 days (200 mg kg^{-1} each day) [42]. We observed efficient recombination but a high mortality rate (date not shown). Therefore we do not recommend this protocol.

In conclusion, which model should be chosen when a strong expression of a transgene is required in adult blood endothelial cells without any concomitant expression in blood cells (Table S1)? Constitutive *Tie2-Cre* mice could be considered but only after wild-type bone marrow transplantation. *Cdh5(PAC)-CreERT2* mice can be used, keeping in mind that they need 10 mg tamoxifen IP (500 mg kg^{-1}) for constant recombination. *Pdgfb-iCreERT2* are good candidates for most blood vessel network investigations, including the brain, but with the exception of liver studies as hepatic sinusoidal endothelial cells are not recombined. Caution should be observed if the transgene of interest is also expressed in hematopoietic cells, and especially if its expression induces hematopoietic progenitor proliferation.

Addendum

B. Kilani conducted and designed experiments, analyzed and interpreted the data and wrote the manuscript. V. Gourdou-Latyszenok, A. Guy, M.L. Bats, C. Peghaire and M. Parrens conducted experiments and analyzed data. M.A. Renault, C. Duplaa, J.L. Villeval and P.E. Rautou analyzed data. T. Couffinhal and C. James designed the experiments, analyzed and interpreted the data and edited the manuscript. All authors discussed the results and commented on the manuscript.

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Disclosures of Conflict of Interests

The authors state that they have no conflict of interests.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Fig. S1. Merged and single channel images showed in figure 2 to address the efficiency of Cre-recombinase expression in various tissues. (A) Constitutive Tie2-Cre;mT/mG mice. (B) Inducible Pdgfb-iCreERT2;mT/mG. (C) Efficiently-recombined Cdh5(PAC)-CreERT2;mT/mG mice. (D) Weakly-recombined Cdh5(PAC)-CreERT2;mT/mG mice. All inducible mice were analysed 5 weeks after tamoxifen induction. Merged and single channel images are shown. Endothelial cells are red stained with a rat anti-CD31 antibody, and co-localization of CD31 and EGFP (green) appears yellow (arrows). CD31+ endothelial cells that are not recombined (EGFP negative) are indicated by an arrowhead. Nucleus, DAPI (blue); tdTomato (purple). Images are representative of at least 3 mice.

Fig. S2. The Cre-recombinase is not expressed in Pdgfb-iCreERT2 positive;mT/mG without tamoxifen induction. Fluorescence confocal imaging of the vasculature of adult retina, liver, heart, lung, kidney and brain CD31+ vessels in inducible Pdgfb-iCreERT2;mT/mG mice 5 weeks after vehicle induction. Merged and single images are shown. Endothelial cells are red stained with a rat anti-CD31 antibody, and co-localization of CD31 and EGFP (green) appears yellow (arrows). Rare CD31+ endothelial cells that are EGFP positive are indicated by an arrow. Note that these cells are both EGFP and Tomato positive, demonstrating that the EGFP expression is not due to Cre mediated recombination. Nucleus, DAPI (blue); tdTomato (purple). Images are representative of at least six mice per group.

Table S1. Summary of advantages and inconvenients of endothelial mouse models for experiments where strong expression of a transgene is required in adult blood endothelial cells (BEC) without concomitant expression in hematopoietic cells.

References

1 Kazmi RS, Boyce S, Lwaleed BA. Homeostasis of hemostasis: the role of endothelium. *Semin Thromb Hemost* 2015; **41**: 549–55.

2 Aghourian MN, Lemarie CA, Bertin FR, Blostein MD. Prostaglandin E synthase is upregulated by Gas6 during cancer-induced venous thrombosis. *Blood* 2016; **127**: 769–77.

3 De Grandis M, Cambot M, Wautier MP, Cassinat B, Chomienne C, Colin Y, Wautier JL, Le Van Kim C, El Nemer W. JAK2V617F activates Lu/BCAM-mediated red cell adhesion in polycythemia vera through an EpoR-independent Rap1/Akt pathway. *Blood* 2013; **121**: 658–65.

4 von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Kollnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, *et al*. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med* 2012; **209**: 819–35.

5 Terry RW, Kwee L, Baldwin HS, Labow MA. Cre-mediated generation of a VCAM-1 null allele in transgenic mice. *Transgenic Res* 1997; **6**: 349–56.

6 Gustafsson E, Brakebusch C, Hietanen K, Fassler R. Tie-1-directed expression of Cre recombinase in endothelial cells of embryoid bodies and transgenic mice. *J Cell Sci* 2001; **114**: 671–6.

7 Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* 2001; **230**: 230–42.

8 Licht AH, Raab S, Hofmann U, Breier G. Endothelium-specific Cre recombinase activity in flk-1-Cre transgenic mice. *Dev Dyn* 2004; **229**: 312–8.

9 Alva JA, Zovein AC, Monvoisin A, Murphy T, Salazar A, Harvey NL, Carmeliet P, Iruela-Arispe ML. VE-Cadherin-Cre-recombinase transgenic mouse: a tool for lineage analysis and gene deletion in endothelial cells. *Dev Dyn* 2006; **235**: 759–67.

10 Oberlin E, El Hafny B, Petit-Cocault L, Souyri M. Definitive human and mouse hematopoiesis originates from the embryonic endothelium: a new class of HSCs based on VE-cadherin expression. *Int J Dev Biol* 2010; **54**: 1165–73.

11 Ulyanova T, Scott LM, Priestley GV, Jiang Y, Nakamoto B, Koni PA, Papayannopoulou T. VCAM-1 expression in adult hematopoietic and nonhematopoietic cells is controlled by tissue-inductive signals and reflects their developmental origin. *Blood* 2005; **106**: 86–94.

12 Tang Y, Harrington A, Yang X, Friesel RE, Liaw L. The contribution of the Tie2+ lineage to primitive and definitive hematopoietic cells. *Genesis* 2010; **48**: 563–7.

13 Lugus JJ, Park C, Ma YD, Choi K. Both primitive and definitive blood cells are derived from Flk-1+ mesoderm. *Blood* 2009; **113**: 563–6.

14 Zovein AC, Hofmann JJ, Lynch M, French WJ, Turlo KA, Yang Y, Becker MS, Zanetta L, Dejana E, Gasson JC, Tallquist MD, Iruela-Arispe ML. Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell* 2008; **3**: 625–36.

15 Forde A, Constien R, Grone HJ, Hammerling G, Arnold B. Temporal Cre-mediated recombination exclusively in endothelial cells using Tie2 regulatory elements. *Genesis* 2002; **33**: 191–7.

16 Gothert JR, Gustin SE, van Eekelen JA, Schmidt U, Hall MA, Jane SM, Green AR, Gottgens B, Izon DJ, Begley CG. Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* 2004; **104**: 1769–77.

17 Monvoisin A, Alva JA, Hofmann JJ, Zovein AC, Lane TF, Iruela-Arispe ML. VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. *Dev Dyn* 2006; **235**: 3413–22.

18 Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, Adams S, Davy A, Deutsch U, Luthi U, Barberis A, Benjamin LE, Makinen T, Nobes CD, Adams RH. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature* 2010; **465**: 483–6.

19 Claxton S, Kostourou V, Jadeja S, Chambon P, Hodivala-Dilke K, Fruttiger M. Efficient, inducible Cre-recombinase activation in vascular endothelium. *Genesis* 2008; **46**: 74–80.

20 Aird WC. Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circ Res* 2007; **100**: 174–90.

21 Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis* 2007; **45**: 593–605.

22 Hasan S, Lacout C, Marty C, Cuingnet M, Solary E, Vainchenker W, Villeval JL. JAK2V617F expression in mice amplifies early hematopoietic cells and gives them a competitive advantage that is hampered by IFNalpha. *Blood* 2013; **122**: 1464–77.

23 James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* 2005; **434**: 1144–8.

24 Descamps B, Sewduth R, Ferreira Tojais N, Jaspard B, Reynaud A, Sohet F, Lacolley P, Allieres C, Lamaziere JM, Moreau C, Dufourcq P, Couffinhal T, Duplaa C. Frizzled 4 regulates arterial network organization through noncanonical Wnt/planar cell polarity signaling. *Circ Res* 2012; **110**: 47–58.
25 Renault MA, Chapouly C, Yao Q, Larrieu-Lahargue F, Vandierdonck S, Reynaud A, Petit M, Jaspard-Vinassa B, Belloc I, Traiffort E, Ruat M, Duplaa C, Couffinhal T, Desgranges C, Gadeau AP. Desert hedgehog promotes ischemia-induced angiogenesis by ensuring peripheral nerve survival. *Circ Res* 2013; **112**: 762–70.
26 Breart B, Ramos-Perez WD, Mendoza A, Salous AK, Gobert M, Huang Y, Adams RH, Lafaille JJ, Escalante-Alcalde D, Morris AJ, Schwab SR. Lipid phosphate phosphatase 3 enables efficient thymic egress. *J Exp Med* 2011; **208**: 1267–78.
27 Briot A, Civelek M, Seki A, Hoi K, Mack JJ, Lee SD, Kim J, Hong C, Yu J, Fishbein GA, Vakili L, Fogelman AM, Fishbein MC, Lusis AJ, Tontonoz P, Navab M, Berliner JA, Iruela-Arispe ML. Endothelial NOTCH1 is suppressed by circulating lipids and antagonizes inflammation during atherosclerosis. *J Exp Med* 2015; **212**: 2147–63.
28 Mikelis CM, Simaan M, Ando K, Fukuhara S, Sakurai A, Amornphimoltham P, Masedunskas A, Weigert R, Chavakis T, Adams RH, Offermanns S, Mochizuki N, Zheng Y, Gutkind JS. RhoA and ROCK mediate histamine-induced vascular leakage and anaphylactic shock. *Nat Commun* 2015; **6**: 6725.
29 Etheridge SL, Roh ME, Cosgrove ME, Sangkhae V, Fox NE, Chen J, Lopez JA, Kaushansky K, Hitchcock IS. JAK2V617F-positive endothelial cells contribute to clotting abnormalities in myeloproliferative neoplasms. *Proc Natl Acad Sci USA* 2014; **111**: 2295–300.
30 Winkler IG, Barbier V, Nowlan B, Jacobsen RN, Forristal CE, Patton JT, Magnani JL, Levesque JP. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat Med* 2012; **18**: 1651–7.
31 Tiedt R, Schomber T, Hao-Shen H, Skoda RC. Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood* 2007; **109**: 1503–6.
32 Calaminus SD, Guitart AV, Sinclair A, Schachtner H, Watson SP, Holyoake TL, Kranc KR, Machesky LM. Lineage tracing of Pf4-Cre marks hematopoietic stem cells and their progeny. *PLoS ONE* 2012; **7**: e51361.
33 Pertuy F, Aguilar A, Strassel C, Eckly A, Freund JN, Duluc I, Gachet C, Lanza F, Leon C. Broader expression of the mouse platelet factor 4-cre transgene beyond the megakaryocyte lineage. *J Thromb Haemost* 2015; **13**: 115–25.
34 Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999; **21**: 70–1.
35 Shahani T, Covens K, Lavend'homme R, Jazouli N, Sokal E, Peerlinck K, Jacquemin M. Human liver sinusoidal endothelial cells but not hepatocytes contain factor VIII. *J Thromb Haemost* 2014; **12**: 36–42.
36 Mehta AK, Majumdar SS, Alam P, Gulati N, Brahmachari V. Epigenetic regulation of cytomegalovirus major immediate-early promoter activity in transgenic mice. *Gene* 2009; **428**: 20–4.
37 Huh WJ, Khurana SS, Geahlen JH, Kohli K, Waller RA, Mills JC. Tamoxifen induces rapid, reversible atrophy, and metaplasia in mouse stomach. *Gastroenterology* 2012; **142**: 21–4.e7.
38 Denk F, Ramer LM, Erskine EL, Nassar MA, Bogdanov Y, Signore M, Wood JN, McMahon SB, Ramer MS. Tamoxifen induces cellular stress in the nervous system by inhibiting cholesterol synthesis. *Acta Neuropathol Commun* 2015; **3**: 74.
39 Gladwin AM, Carrier MJ, Beesley JE, Lelchuk R, Hancock V, Martin JF. Identification of mRNA for PDGF B-chain in human megakaryocytes isolated using a novel immunomagnetic separation method. *Br J Haematol* 1990; **76**: 333–9.
40 Padron-Barthe L, Temino S, Villa dCC, Carramolino L, Isern J, Torres M. Clonal analysis identifies hemogenic endothelium as the source of the blood-endothelial common lineage in the mouse embryo. *Blood* 2014; **124**: 2523–32.
41 Park EJ, Sun X, Nichol P, Saijoh Y, Martin JF, Moon AM. System for tamoxifen-inducible expression of cre-recombinase from the Foxa2 locus in mice. *Dev Dyn* 2008; **237**: 447–53.
42 Maillard I, Chen YX, Friedman A, Yang Y, Tubbs AT, Shestova O, Pear WS, Hua X. Menin regulates the function of hematopoietic stem cells and lymphoid progenitors. *Blood* 2009; **113**: 1661–9.