

Supplementary Note.

Characterization of blood and bone marrow Tie2p/e-GFP+ cells. *Blood and bone marrow samples were harvested and processed for FACS analysis as it follows. For immunostaining, 10^5 to 10^6 cells were blocked in 5% rat serum (StemCell Technologies), 2% fetal bovine serum in phosphate buffered saline for 15 min at 4°C. After blocking, RPE-conjugated antibodies (IgG2a kappa isotype control, clone R35-95; CD45 (Ly-5), clone 30-F11; CD19, clone 1D3; Sca-1 (Ly-6A/E), clone E13-161.7; c-Kit (CD117), clone ack45, all by BD PharMingen, San Diego, CA and CD11b (Mac-1), clone MI/70.15, by Serotec, Raleigh, NC) were added to a final concentration of 1-5 µg/ml and the cells incubated for 30 min at 4°C, then washed, stained with 30_M propidium iodide (Beckton Dickinson) and analysed by three-color flow cytometry.*

From 68% to 96% (average 83%, n =8) and from 75% to 92% (average 82%, n =7) of the blood leukocytes expressed GFP to high levels (average M.F.I. 790 and 1200) in PGKp-BMT nude and immunocompetent mice, respectively. Expression of the CMVp vector was more heterogeneous among the total cells (9%-53% GFP+, average 25%, M.F.I.=350, n =7). GFP fluorescence was similarly prevalent in the red blood cells of all mice described above. Only 1-4% (average 2%, n = 20) and 2-6% (average 4%, n = 9) of the blood leukocytes were weakly positive for GFP expression (average M.F.I. 52 and 44) in Tie2p/e-BMT nude and immunocompetent mice, respectively. FACS analysis of the peripheral blood of Tie2p/e-BMT mice revealed that the low-frequency GFP positive cells were of hemopoietic origin (CD45+) and expressed surface markers typical of myeloid (CD11b+) but not lymphoid (CD19-) lineage. The physical distribution of the GFP+ cells in the FACS dot plot suggested that the circulating Tie2p/e-GFP+ cells might represent a sub-population of monocytic cells. Expression in the BM cells was consistent with that measured in the peripheral blood for all types of BMT mice. In the BM of Tie2p/e-BMT mice, a significant percentage of the small

fraction of weakly GFP positive cells, that uniformly expressed the CD45 marker, also expressed the stem and progenitor cell-specific markers c-Kit and Sca-1. These findings are in agreement with previous reports of Tie2 receptor expression in subsets of hematopoietic stem cells^{6,16,17}. Moreover, the low-level expression observed in a small fraction of BM cells became undetectable upon proliferation and differentiation of the cells in liquid culture and in clonogenic assays (not shown).

Lack of contribution of BM-derived cells to tumor EC. We established the following criteria to properly discriminate GFP+ EC from GFP+ peri-endothelial cells and leukocytes in *diapedesis*. The criteria were established according to the co-localization of GFP and CD31 staining observed in vessels of transgenic mice expressing GFP under the control of the Tie2 promoter/enhancer or the CMV/ β -actin enhancer/promoter.

1. *GFP+ EC had to display full co-localization of GFP and CD31 staining.* In tumors and tissues of transgenic mice expressing GFP under the control of the Tie2 promoter/enhancer or CMV/ β -actin enhancer/promoter, CD31 and GFP staining fully co-localize in EC of blood vessels. When the EC nucleus is comprised in the section, CD31 staining localizes mostly at the cell membrane, overlapping and surrounding GFP staining (and the cell nucleus) along with the entire perimeter of the cell (see Tie2TgN in Fig. 2e). In these control GFP+ CD31+ EC, co-localization of GFP and CD31 staining is maintained in all the serial confocal planes in which individual cells are analyzed, and no divergence of the two signals is observed. GFP+ cells intimately associated with CD31+ EC at the adluminal side of blood vessels and GFP+ cells in diapedesis or bound to the luminal side of blood vessels may display partial co-localization of GFP and CD31 staining (Fig. 2f). For instance, if a GFP+ cell partially wraps a GFP-

CD31+ EC, the two cells may be mistaken for a single GFP+ EC with its membrane partially positive for CD31 staining (Fig. 2g). Usually, co-localization of the two markers in the same cell can be ruled out at high-power magnification (Fig. 2f), by the analysis of serial confocal planes, where the two signals may diverge, or as a result of the identification of two cell nuclei. In such cases, CD31 staining does not completely surround the GFP+ cell, in contrast to true GFP+ EC (Fig. 2g). Cells in diapedesis can be virtually incorporated in the EC layer. However, if only the cell in diapedesis is GFP+, GFP and CD31 signals do not co-localize at high-power magnification. In addition, GFP+ erythrocytes may bind to CD31+ EC of blood vessels and produce spotted, false double-positive signals. Perfusion of the animal helps to reduce this phenomenon.

2. *Lack of CD45 staining. CD31+ EC do not express the pan-leukocyte marker CD45 and other hematopoietic markers, such as CD11b (a myeloid lineage marker). Putative GFP+ EC should express CD31 and not CD45 or CD11b. Triple staining of GFP, CD31 and CD45 or CD11b can allow discrimination of hematopoietic cells tightly associated with EC or in diapedesis.*

Frequency of BM-derived, Tie2p/e-GFP+ EC in tumor vessels.

To determine the frequency of the Tie2p/e-GFP+ cells that were incorporated in blood vessels as bona fide EC, six different tumor sections from each of 26 tumors were analyzed (Exp.cIV: 4 nude Tie2p/e-BMT mice, each mouse challenged with one TS/A and one B16 tumor, grown for 3 weeks; Exp.cVI: 2 nude Tie2p/e-BMT mice, each one challenged with a TS/A tumor, grown for 1 week; Exp.cVIII: 4 nude Tie2p/e-BMT mice, each one challenged with one TS/A tumor, grown for 2-3 weeks; Exp.cXVI: 6 C57/BL6 Tie2p/e-BMT mice, each one challenged with one LLC and one B16 tumor, grown for 2-3 weeks). Four sections/tumor

were double-immunostained for GFP and CD31, two sections/tumor were triple-immunostained for GFP, CD31 and CD11b. The whole tumor section was scanned at x200 and 3-10 x200 fields that contained more abundant Tie2p/e-GFP+ cells were selected and digitally acquired. The majority of the Tie2p/e-GFP+ cells were identified at the periphery of tumors, in correspondence with highly vascularized regions. In some of the regions at the boundaries with the host tissues, the Tie2p/e-GFP+ cells represented a significant fraction of the CD45+ or CD11b+ cells (up to 30% of the total leukocytes). In the inner mass of the tumors, the Tie2p/e-GFP+ cells represented a small fraction, usually 0-1% of the total tumor-infiltrating hematopoietic cells. The Tie2p/e-GFP+ cells that appeared as possible CD31+ EC were then analyzed at higher magnification ($\geq x1000$) to assess or exclude the co-localization of GFP, CD31 and, eventually, CD11b staining, as explained above. We estimated that an average of 200-250 vessels/mm² are present in the highly vascularized regions of either tumor (see also Fig. 3c), where the Tie2p/e-GFP+ cells were more abundant. We calculated that at least 60,000 blood vessels (including small capillaries and larger vessels) were screened for the presence of GFP+ EC. We could detect and document 7 bona fide Tie2p/e-GFP+ cells that met one of the criteria mentioned above (double-staining for GFP and CD31). In the sections analyzed by triple immunostaining, we documented 3 Tie2p/e-GFP+ CD31+ cells that were also CD11b+. In conclusion, the frequency of Tie2p/e-GFP+ CD31+ was approximately 1 in 6,000 blood vessels.

We obtained similarly negative findings from analysis of a smaller collection of tumors grown in mice transplanted with transgenic donor cells expressing GFP under the Tie2 promoter/enhancer (Exp cXVIII: 6 nude Tie2TgN-BMT mice, each one challenged with one TS/A and one N202 tumor, grown for 1, 2 or 4 weeks). In fact, in the sections analyzed (4 sections stained for GFP and CD31 and 4 sections stained for GFP and CD45) from each

tumor grown in Tie2TgN-BMT mice, we could only detect isolated or peri-vascular CD45+ GFP+ cells and no CD31+ GFP+ EC.

Frequency of BM-derived EC in tumor vessels.

The same criteria were used for the identification of BM-derived GFP+ EC in mice transplanted with BM Lin- cells transduced by the PGKp-GFP vector (PGKp-BMT) or derived from transgenic mice ubiquitously expressing GFP under the CMV/ β -actin enhancer/promoter (ACTbTgN-BMT). Because of the widespread infiltration of GFP+ cells in tumors grown in these mice, we randomly selected and then carefully analyzed 1,199 individual vessels for the presence of GFP+ EC. Each blood vessel (a small capillary or a larger vessel) was analyzed at $\geq \times 1000$ magnification (as described above) and the total number of GFP+ cells present in the field counted. We analyzed a total of 42 tumor sections from a collection of 21 tumors grown in the two types of BMT mice (Exp.cVII, 8 nude PGKp-BMT mice challenged with 2 TS/A, 3 LLC and 3 B16 tumors, grown for 2-3 weeks; Exp.cXII, 3 nude ACTbTgN-BMT mice challenged with 3 TS/A tumors and 6 C57/BL6 ACTbTgN-BMT mice challenged with 3 LLC and 3 B16 tumors, all grown for 1-4 weeks; Exp.cXIV, 4 C57BL/6 PGKp-BMT mice challenged with 2 LLC and 2 B16 tumors, grown for 2-4 weeks), as indicated in the following tables. In all the tumor sections and blood vessels carefully examined, we could not detect any bona fide GFP+ EC that met the above-mentioned criteria for the co-localization of GFP and CD31 markers.

PGKp-BMT

Tumor type	Number of tumors analyzed	Total tumor sections/ x1000 tumor fields analyzed	Individual vessels analyzed	Total GFP+ cells	<i>GFP+/CD31+ EC</i>
LLC/3LL	3 ^N , 2 ^{IC}	10/30	170	770	0
B16/BL	3 ^N , 2 ^{IC}	10/38	184	331	0
TS/A	2 ^N	4/31	168	1861	0

ACTbTgN-BMT

Tumor type	Number of tumors analyzed	Total tumor sections/ x1000 tumor fields analyzed	Individual vessels analyzed	Total GFP+ cells	<i>GFP+/CD31+ EC</i>
LLC/3LL	3 ^{IC}	6/34	205	876	0
B16/BL	3 ^{IC}	6/25	98	312	0
TS/A	3 ^N	6/39	374	2736	0

^N = nude mouse

^{IC} = immunocompetent mouse

Six weeks after the transplant, FACS analysis showed that *PGKp-tk-BMT* nude mice (n=16) expressed GFP in the vast majority of the blood leukocytes (average 68% GFP+ cells, note that GFP expression is IRES-dependent in these mice), while less than 2% of the cells were slightly positive for GFP in *Tie2p/e-tk-BMT* nude (n=16) and C57BL/6 immunocompetent (n=11) mice. When the mice were killed at the end of the experiment, BM cells from three nude mice of each group were plated in a methylcellulose-based medium either containing or not GCV (10 µg/ml). Twelve days after plating, hematopoietic colonies were counted and harvested for PCR analysis of vector sequence.

GCV did not inhibit the growth of hematopoietic colonies from the BM of Tie2p/e-tk-BMT and control tk-negative mice in CFC assays. On the contrary, colony growth was almost completely inhibited from the BM of PGKp-tk-BMT mice that had not received GCV *in vivo*. Unexpectedly, we observed outgrowths of GCV-resistant colonies from the BM of PGKp-tk-BMT mice that were treated with GCV. This observation and the lack of GFP fluorescence in the CFC (data not shown) indicated that selection of tk-negative, GCV-resistant hematopoietic progenitor cells occurred upon GCV treatment *in vivo*. This observation was in agreement with the finding that, despite the broad expression of the suicide gene in the hematopoietic system of untreated PGKp-tk-BMT mice and the efficient elimination of transgene-expressing cells following GCV administration, histology on paraffin-embedded BM samples taken after 12 days of GCV treatment failed to reveal major signs of myelotoxicity (data not shown). Notably, at the same time peripheral blood cell counts were significantly decreased in these mice. The finding of an *in vivo* selection process may explain the presence of tumor-infiltrating CD45+ GFP-leukocytes in samples harvested from the GCV-treated PGKp-tk-BMT mice. GCV-induced myelosuppression in PGKp-tk-BMT mice most likely was exhaustive during the first days of treatment, when tumor growth was almost completely inhibited. Later on, the selection process may have rescued hematopoiesis and allowed the growth, even though significantly delayed and slower, of the tumor graft in GCV treated mice.