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# Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells

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**Angiogenic tumor vessels are promising targets for the activity and the selective delivery of cancer therapeutics[1,2]. The bone marrow contributes different cell types to the tumor stroma, including hematopoietic cells[3,4] and, as recently suggested, vascular endothelial cells (ECs)[5]. Thus, transplantation of genetically modified bone marrow progenitors may represent a vehicle for the transport of gene therapy to tumors. We transduced bone marrow progenitors with lentiviral vectors expressing genes from transcription-regulatory elements of *Tie2/Tek* gene[6]. When tumors were grown in the transplanted mice, the new vector marked a distinct hematopoietic population that 'homed' to the tumor and closely interacted with vascular ECs at the tumor periphery. These Tie2-expressing mononuclear (TEM) cells had a distinguishable phenotype and were present selectively at angiogenic sites. Unexpectedly, we did not find bone marrow–derived ECs in tumor vessels when we transplanted bone marrow progenitors constitutively expressing a marker gene from the *Tie2* or ubiquitously active promoters. By delivering a 'suicide' gene, we selectively eliminated the TEM cells and achieved substantial inhibition of angiogenesis and slower tumor growth without systemic toxicity. Thus, TEM cells may account for the proangiogenic activity of bone marrow–derived cells in tumors, may represent a new target for drug development and may provide the means for selective gene delivery and targeted inhibition of tumor angiogenesis.**

To specifically target exogenous genes to tumor angiogenesis using bone marrow–derived cells, we generated lentiviral vectors with EC-specific expression. We cloned promoter and enhancer sequences from the *Tie2/Tek* gene[7], which is preferentially expressed in ECs and encodes an angiopoietin receptor tyrosine kinase, into a late-generation, self-inactivating lentiviral vector[8], and called the resultant vector Tie2p/e (**Fig. 1a**). To evaluate the specificity of expression of the new vector, we transduced a wide panel of cell lines and primary cultures from different tissues and compared expression of green fluorescent protein (GFP) of the Tie2p/e vector with that of lentiviral vectors carrying the ubiquitously expressed phosphoglycerate promoter (PGKp)[8] and cytomegalovirus promoter (CMVp; data not shown)[9]. Analysis of GFP expression in cells with similar integration amounts of each vector showed that the Tie2p/e vector was efficiently and selectively expressed in ECs *in vitro* (**Supplementary Fig. 1** online). To test the vectors *in vivo*, we injected them directly into mammary tumor (TS/A) allografts grown subcutaneously in nude mice (**Supplementary Fig. 2** online) and showed targeted and efficient expression of the Tie2p/e vector in tumor ECs. In addition to ECs, the Tie2p/e vector targeted gene expression to a subset of stromal cells expressing the pan-leukocyte marker CD45 and localized mainly at the tumor periphery.

We next performed bone marrow transplantation (BMT) experiments. We transduced mouse bone marrow–derived lineage-negative progenitors and achieved up to 95% transduction and comparable gene transfer with all vectors (**Fig. 1b** and data not shown). We injected the transduced cells into mice lethally irradiated to allow full engraftment of the transplanted cells and obtained Tie2p/e-BMT, PGKp-BMT and CMVp-BMT mice. From 12 to 24 weeks after transplantation, we used clonogenic assays to detect vector-specific sequences in bone marrow–derived colony-forming cells and confirmed that there was comparably high engraftment of the donor cells transduced with each vector (**Fig. 1c**). Fluorescence-activated cell sorting (FACS) analysis showed that PGKp-BMT and CMVp-BMT mice had strong GFP expression in all blood cell lineages and in the bone marrow, whereas only a small fraction of the blood leukocytes and marrow cells were weakly GFP-positive in Tie2p/e-BMT mice (**Fig. 1d**). The circulating Tie2p/e-GFP–positive cells were hematopoietic and of myeloid lineage ($CD45^+CD11b^+CD19^-$; **Fig. 1e** and **Supplementary Note** online).

From 4 to 48 weeks after transplantation, we established different tumor grafts in the transplanted mice by subcutaneous injection of mammary carcinoma (TS/A) or Lewis lung carcinoma (LLC) or melanoma (B16) cells (**Fig. 1f**). Immunofluorescence staining and confocal microscopy of carcinomas grown in PGKp-BMT mice showed extensive infiltration of GFP-positive cells throughout the tumor. The GFP-positive cells were $CD45^+$ and most of them expressed the macrophage-specific F4/80 marker. GFP was also expressed in granulocytes identified by expression of the Gr-1/Ly-

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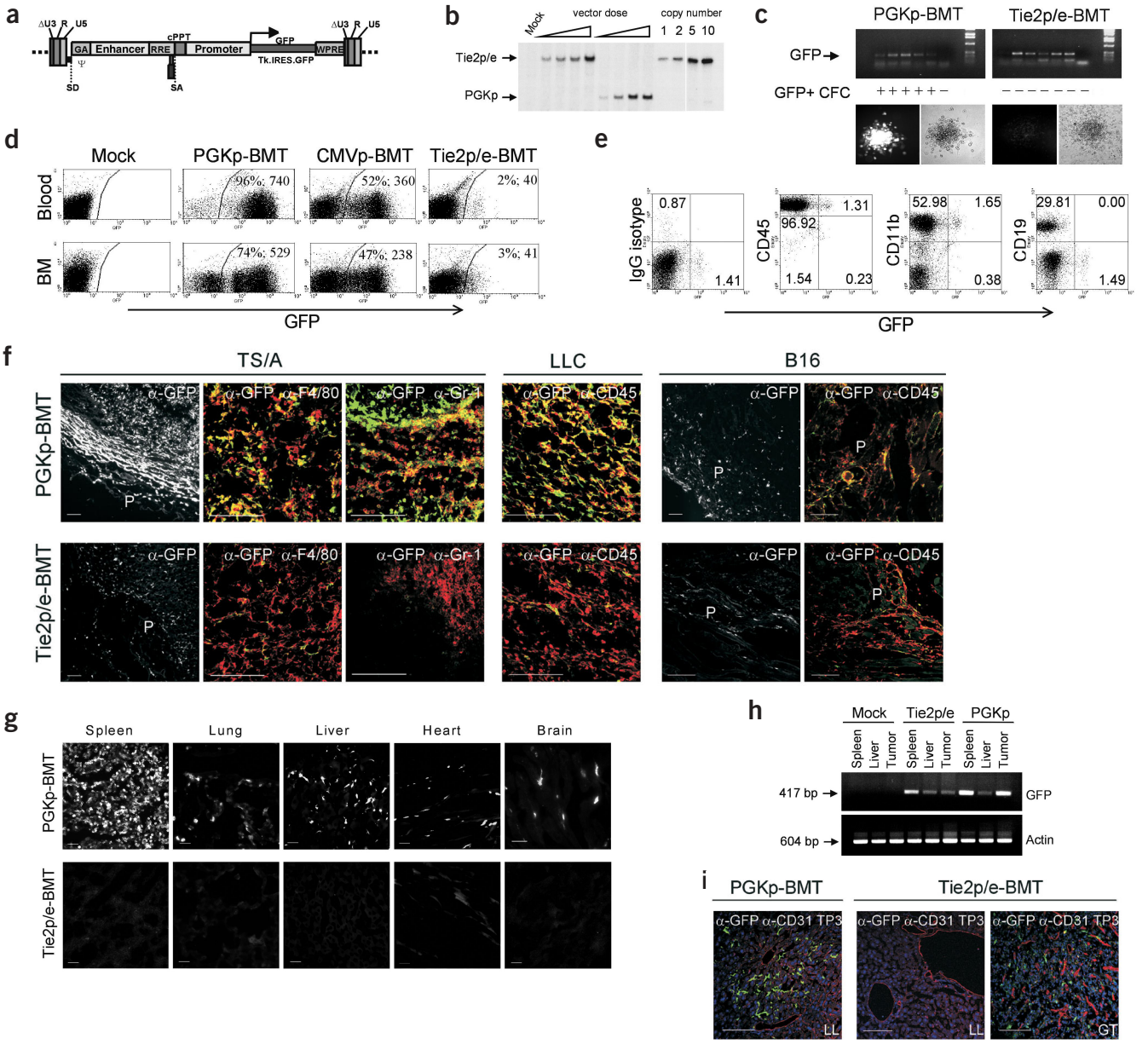


**Figure 1** Recruitment of transduced bone marrow–derived cells to the tissues of transplanted mice and restricted expression of the Tie2p/e vector. (**a**) The self-inactivating Tie2p/e lentiviral vector (proviral form). Transcription of GFP or tk-IRES-GFP was driven by the *Tie2* promoter and enhancer sequences. ΔU3, R and U5, LTR regions with deletion in U3; SD and SA, splice donor and acceptor sites; Ψ, encapsidation signal including the 5′ portion of *gag* gene (GA); cPPT, central polypurine tract; RRE, rev response element; WPRE, woodchuck hepatitis virus post-transcription regulatory element. (**b**) Southern blot analysis of bone marrow–derived lineage-negative cells mock-transduced or transduced with increasing doses (wedges) of Tie2p/e and PGKp lentiviral vectors. These had comparable vector integration, with the highest dose yielding three to five copies per cell. (**c**) PCR analysis of GFP sequence (top) and fluorescence microscopy (scored as fluorescence-positive (+) or fluorescence-negative (–)) of hematopoietic colonies from the bone marrow of transplanted mice. These had a similar frequency of transduced colony-forming cells (CFC; average, 69%; $n = 18$ from three mice per vector) and no expression by the Tie2p/e lentiviral vector. Analysis of two representative mice, with fluorescence and phase-contrast pictures of representative colonies shown (bottom). (**d**) FACS analysis of GFP expression in the blood and bone marrow (BM) of four representative mice 12 weeks after BMT. Quadrants include the percentage and mean fluorescence intensity of GFP-positive cells. The control mouse was transplanted with mock-transduced cells. (**e**) FACS analysis. The circulating GFP-positive cells in a representative Tie2p/e-BMT mouse 48 weeks after BMT were hematopoietic ($CD45^+$) and of myeloid lineage ($CD11b^+CD19^-$). Quadrants include the percentage of GFP-positive cells. (**f**) Confocal immunofluorescence analysis of carcinomas (TS/A and LLC) and melanomas (B16) grown for 2–3 weeks in nude (TS/A; B16, left column) or C57BL/6 (LLC; B16, right column) BMT mice and immunostained for GFP (grayscale or green), F4/80, Gr-1 or CD45 (red). Yellow, double-positive cells. The Tie2p/e vector marked a subset of $CD45^+$ cells at the tumor periphery (P). Representative photos of tumors from 6–20 BMT mice per vector shown here. Scale bar = 120 μm. (**g**) GFP immunostaining of organs from transplanted mice. The Tie2p/e vector was not expressed in these tissues despite the considerable bone marrow contribution shown by GFP-positive cells in PGKp-BMT mice. Representative findings from three or more mice per vector shown here. Scale bar = 30 μm. (**h**) Semi-quantitative PCR for the GFP sequence on spleen, liver and tumor DNA. There was comparable integration of both vectors in each organ of the indicated BMT mice. Mock, same as in **d**. (**i**) Immunostaining for GFP (green) and CD31 (red) and nuclear DNA staining by TO-PRO-3 (TP3, blue). Tie2p/e-GFP–positive cells were present in the granulation tissue (GT) that surrounded regenerating liver lobules (LL) of C57Bl/6 BMT mice 1 week after partial hepatectomy. Representative photos of livers from three BMT mice per vector shown here. α-, antibody to. Scale bar = 120 μm.

6G marker. In carcinomas from Tie2p/e-BMT mice, the GFP-positive cells were much fewer, expressed CD45 and were localized mainly at the tumor periphery. Most F4/80$^+$ macrophages and Gr-1$^+$ granulocytes infiltrating these tumors did not express the Tie2p/e vector. In melanomas, we found much less leukocyte infiltration. The frequency and distribution of GFP-positive cells were similar in carcinomas and melanomas grown in Tie2p/e-BMT mice. These results indicated that expression of the Tie2p/e vector was restricted to a population of bone marrow–derived hematopoietic cells distinguishable from common macrophages by their recruitment and distribution pattern in the tumors. We obtained similar results in nude and immunocompetent mice and in tumors analyzed 1–4 weeks after implantation.

We also analyzed organs from the transplanted mice (**Fig. 1g**). As expected, the spleens of PGKp-BMT and CMVp-BMT mice were replenished with GFP-positive hematopoietic cells. Bone marrow–derived GFP-positive cells were also abundant in the liver, lung, brain and heart of these mice. Most GFP-positive cells were F4/80$^+$ and were identified as Kupffer cells in the liver, alveolar macrophages in the lung and microglia in the brain (data not shown). Despite the important contribution of bone marrow to the host tissues, we found GFP expression only in the tumors in Tie2p/e-BMT mice. As expected, semi-quantitative PCR showed that there was comparable vector integration in the spleens and livers of Tie2p/e-BMT and PGK-BMT mice (**Fig. 1h**).

We next investigated the recruitment of Tie2p/e-expressing bone marrow–derived cells to sites of nontumor angiogenesis. At 12–48 weeks after BMT, we did partial hepatectomies in PGKp-BMT, CMVp-BMT and Tie2p/e-BMT mice and analyzed the livers 7–10 days later (**Fig. 1i**). We found several Tie2p/e-GFP-positive cells in the granulation tissue surrounding the regenerating hepatic lobules. These cells had surface markers and a spatial relationship with newly formed vessels similar to those in the tumors (discussed below). We obtained similar findings in nude (data not shown) and immunocompetent mice. Thus, vector expression in Tie2p/e-BMT mice was specifically restricted to a subset of bone marrow–derived cells homing to the tumor and to other sites of angiogenesis.

To characterize the Tie2p/e-GFP-positive cells in the tumors, we assessed expression of several cell-type-specific markers (**Fig. 2a**). At the tumor periphery, the GFP-positive cells appeared as small or elongated mononuclear cells, often tightly associated in cord-like or laminar structures. These GFP-positive cells were identified as CD45$^+$ (**Fig. 1f**), Gr-1$^-$ (data not shown), CD11b$^+$CD31$^-$ hematopoietic cells and were intermixed with GFP-negative CD11b$^-$CD31$^+$ ECs. Both GFP-positive hematopoietic cells and GFP-negative ECs in these structures expressed Sca-1/Ly-6A/E, Tie2 and, less homogeneously, CD34. Toward the inner tumor, the GFP-positive cells were fewer, expressed CD45 and CD11b and were often associated with small blood vessels dispersed within the parenchyma (**Fig. 2b**). In the inner tumor, expression of Sca-1 and CD34 (data not shown) was restricted to vascular ECs. The Tie2p/e GFP-positive cells did not express the smooth muscle actin marker of pericytes (data not shown). We found a similar distribution of CD45$^+$CD31$^-$ GFP-positive cells in the peripheral regions of tumors grown in mice transplanted with transgenic bone marrow lineage–negative cells expressing GFP under the *Tie2* promoter/enhancer[10] (Tie2TgN-BMT; **Fig. 2c**), indicating concordant expression of the Tie2p/e vector and a Tie2p/e-GFP transgene. Thus, expression of the Tie2p/e vector in bone marrow–derived cells targeted a population of mononuclear cells recruited to the tumor periphery that expressed a distinctive combination of surface markers (CD45, CD11b, Tie2, Sca-1 and CD34) and assembled a stromal framework in close association with ECs. These features indicated their possible involvement in angiogenesis.

Unexpectedly, we identified only rare Tie2p/e-GFP–positive CD31$^+$ ECs in tumor vessels (1 in 6,000 vessels examined; **Fig. 2b**, arrowhead, and **Supplementary Note** online), indicating trace amounts of EC engraftment by the transduced or transgenic bone marrow. These rare GFP-positive CD31$^+$ cells also expressed the myeloid marker CD11b, a feature that distinguished them from the neighboring GFP-negative CD31$^+$ ECs (**Fig. 2d**), possibly indicating sporadic fusion between bone marrow–derived cells and ECs. Although the Tie2p/e vector and the Tie2p/e-GFP transgene were efficiently expressed in ECs (**Fig. 2e** and **Supplementary Figs. 1** and **2** online), we also analyzed tumors grown in PGKp-BMT and CMVp-BMT mice and mice transplanted with transgenic bone marrow lineage–negative cells expressing GFP under control of the CMV/β-actin enhancer/promoter[11] (ACTbTgN-BMT). We did not find GFP-positive ECs in any of the 1,200 tumor vessels analyzed (**Fig. 2e–g** and **Supplementary Note** and **Supplementary Fig. 3** online). We found several vessel-associated GFP-positive cells that expressed CD45 and CD11b, most likely representing leukocytes bound to the endothelium, in diapedesis or intimately associated to the adluminal side of ECs. We obtained these findings in TS/A and LLC carcinomas and in B16 melanomas examined early or late in their growth and that were implanted 4–48 weeks after BMT in nude or immunocompetent mice. The discrepancy between these findings and previous reports of significant bone marrow contribution to tumor ECs[12–15] may be due to differences in the experimental settings (source of stem cells and tumor models) or in the techniques used to identify bone marrow–derived ECs (immunofluorescence for multiple markers and confocal microscopy versus X-gal staining and immunohistochemistry).

To investigate the function of the Tie2p/e vector–targeted cells in angiogenesis and tumor growth, we devised a 'suicide' gene approach based on the herpes simplex virus thymidine kinase (tk)–ganciclovir (GCV) system. We constructed lentiviral vectors expressing a bicistronic tk and GFP cassette containing an internal ribosome entry site (IRES). We transplanted mice with lineage-negative cells transduced with Tie2p/e-tk-IRES-GFP, PGKp-tk-IRES-GFP and control Tie2p/e-GFP vectors. At 8 weeks after the transplant, we left mice untreated or treated them with GCV (100 mg/kg daily) starting before or after tumor implantation (**Fig. 3a**). There was a considerable delay in the appearance of tumors and slower tumor growth in GCV-treated Tie2p/e-tk-BMT and PGKp-tk-BMT mice compared with that of untreated mice and GCV-treated Tie2p/e-BMT mice. FACS analysis showed robust clearance of the GFP-positive cells in the blood and bone marrow (80% and 95% reduction, respectively) in the treated tk-BMT mice. At the end of the treatment, tumors grown in GCV-treated Tie2p/e-tk-BMT and PGKp-tk-BMT mice were significantly smaller in volume ($P < 0.002$ for TS/A and $P < 0.01$ for LLC) and weight (data not shown) than those grown in untreated or GCV-treated control Tie2p/e-BMT mice. CD31 staining of tumor sections showed a much lower vascular density in GCV-treated Tie2p/e-tk-BMT and PGKp-tk-BMT mice than in the controls ($P < 0.005$) and complete absence of infiltrating GFP-positive cells (**Fig. 3b,c**). As expected, we found myelotoxicity in GCV-treated PGKp-tk-BMT mice that had lower platelet counts, hematocrit and hemoglobin. We found no signs of myelotoxicity in GCV-treated Tie2p/e-tk-BMT and control mice (data not shown). GCV did not inhibit the growth of hematopoietic colonies from the bone marrow of Tie2p/e-tk-BMT and control thymidine kinase–negative mice in colony-forming cell assays. In contrast, colony growth was almost

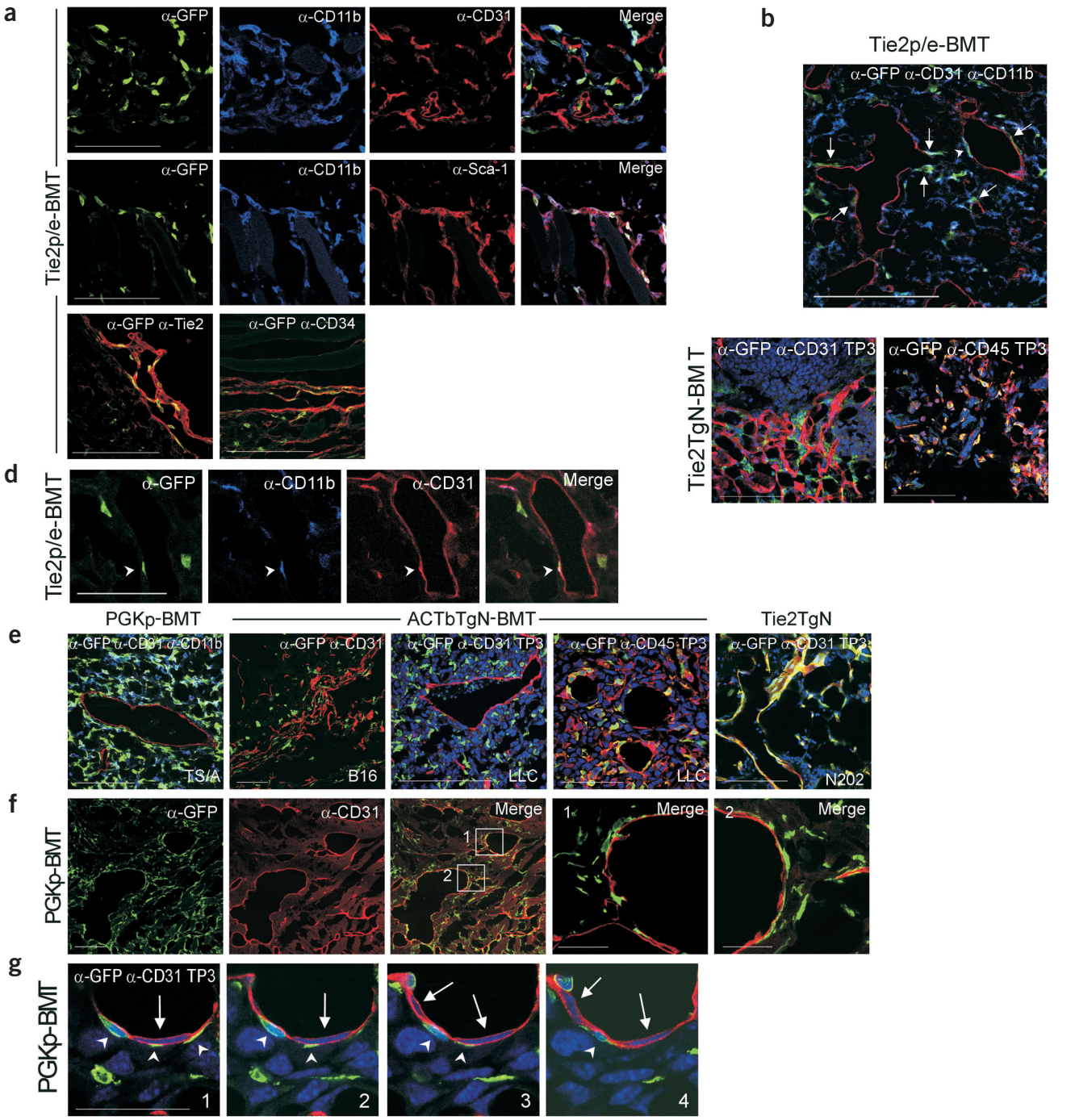


**Figure 2** The Tie2p/e-GFP-positive cells in tumors were hematopoietic with a distinguishable phenotype, closely interacting with but distinct from ECs. Confocal double and triple immunofluorescence analyses of tumors grown in BMT mice and stained for GFP (green), CD11b or TP3 (blue) and CD31, Sca-1, Tie2, CD34 or CD45 (red). Fluorescent signals shown individually or after merging. (**a**) $CD11b^+CD31^-$ GFP-positive cells intermixed with $CD11b^-CD31^+$ GFP-negative ECs at the periphery of a TS/A carcinoma. Both cell populations expressed Sca-1, Tie2 and, less homogeneously, CD34. Scale bar = 120 μm. (**b**) $CD11b^+CD31^-$ GFP-positive cells associated with $CD31^+$ endothelium in the inner tumor mass (arrows). Arrowhead, a rare GFP-positive $CD31^+CD11b^+$ cell. Photos in **a** and **b** are representative of 26 carcinomas and melanomas examined 1–4 weeks after inoculation into nude or C57BL/6 BMT mice. Scale bar = 120 μm. (**c**) GFP-positive, $CD31^-$ (left) and $CD45^+$ (right) cells similar to those in **a** at the periphery of a TS/A carcinoma grown in a Tie2TgN-BMT mouse. Representative photos from six TS/A and six N202 tumors are shown. Scale bar = 120 μm. (**d**) GFP, CD11b and CD31 were coexpressed in the same cell in a TS/A carcinoma of a Tie2p/e-BMT mouse (arrowhead), as shown in these high-magnification photos. Scale bar = 60 μm. (**e**–**g**) Bone marrow–derived cells did not contribute true ECs to tumor vessels. Almost all GFP-positive cells in the tumors in **e** grown in PGKp-BMT and ACTbTgN-BMT mice were $CD31^-$ and expressed the hematopoietic markers CD11b and CD45. In contrast, the $CD31^+$ ECs of a N202 carcinoma grown in a Tie2p/e-GFP transgenic mouse (Tie2TgN; **e**) were GFP-positive. GFP-positive cells associated with tumor vessels at the periphery of a melanoma appeared as GFP-positive, $CD31^+$ ECs at low power. However, GFP and CD31 staining diverged at high magnification (boxes 1 and 2) and in serial confocal planes acquired along the *z*-axis (**g**; 1 μm/step). A GFP-positive $CD31^-$ cell (**g**; arrowheads) was wrapped around two adjacent GFP-negative $CD31^+$ ECs (**g**; arrows). α-, antibody to. Scale bar = 120 μm (**e** and **f**) or 30 μm (**g** and boxes in **f**).

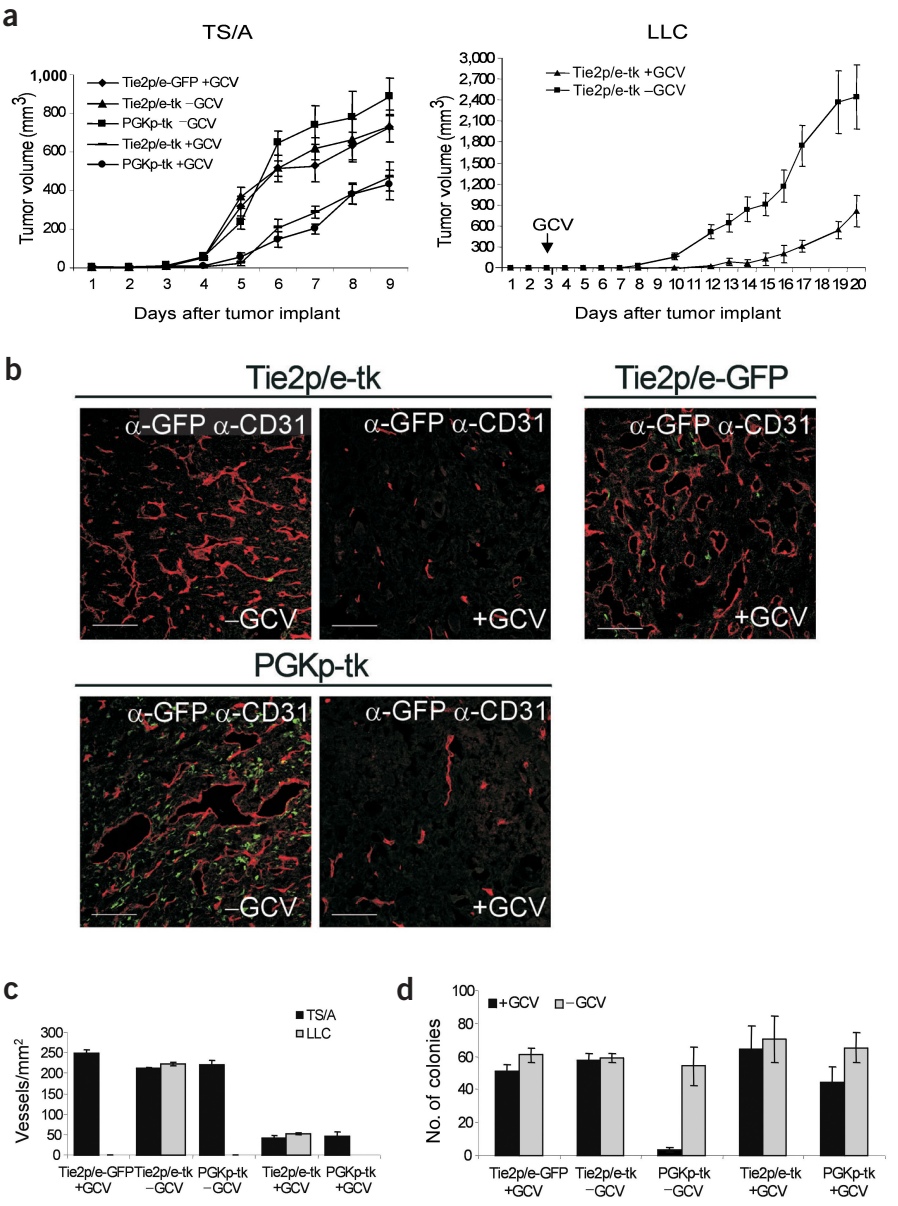


**Figure 3** Selective *in vivo* elimination of the Tie2p/e-vector-targeted cells inhibited tumor angiogenesis and slowed tumor growth. (**a**) Tumor growth (average volume ± s.e.m. in nude mice (left; $n = 10$ tumors per group) and C57BL/6 mice (right; $n = 5$–6) transplanted with bone marrow cells transduced with vectors and treated daily with GCV (+GCV) or left untreated (–GCV). (**b**) Immunostaining for GFP (green) and CD31 (red) of TS/A tumor sections from BMT mice. Representative sections from three mice per group are shown. α-, antibody to. Scale bar = 120 μm. (**c**) Vascular density (average number of vessels/mm$^2$ ± s.e.m.) in TS/A and LLC tumors ($n$ = three tumors per group). (**d**) Colony-forming cell assays, performed in the presence (black bars) or absence (gray bars) of GCV, from BMT mice previously treated with GCV *in vivo* or left untreated (average of duplicate samples from three mice per group ± s.e.m.).

completely inhibited in the bone marrow of PGKp-tk-BMT mice that had not received GCV *in vivo* (**Fig. 3d** and **Supplementary Note** online). PCR analysis of individual colonies showed similarly high engraftment in all groups of untreated mice (67–83% transgene positive; $n = 12$ per group). These results indicated that the elimination of Tie2p/e vector–targeted cells substantially inhibited angiogenesis and slowed tumor growth without obvious effects on hematopoiesis. The extent of tumor inhibition was similar to that obtained with the ubiquitously expressed PGKp vector, indicating that the subset of bone marrow–derived cells expressing the Tie2p/e vector was responsible for most bone marrow–dependent proangiogenic activity in the tumors studied.

During embryogenesis, hematopoietic cells expressing the Tie2 receptor interact with ECs and promote angiogenesis and vascular remodeling[6,16,17]. In adult mice and humans, 'hot spots' of vascular Tie2 expression have been reported at the periphery of invasive tumors[18–20]. In mouse tumor models, bone marrow–derived monocytes and inflammatory cells have been shown to contribute to tumor angiogenesis[14,21,22]. Blocking Flt1, the vascular endothelial growth factor receptor expressed by ECs, monocytes and macrophages[23], results in decreased recruitment of perivascular Flt1$^+$ myeloid cells in tumors and inhibits tumor growth[14,22]. The subset of bone marrow–derived cells we described here and that specifically expressed the Tie2p/e vector or a Tie2p/e-GFP transgene (TEM cells) may have been previously observed along with, but not discriminated from, ECs, perivascular myeloid cells and tumor-associated macrophages at the periphery of tumors and may have been responsible for the reported vascular growth–promoting activities. Indeed, by selectively eliminating the small fraction of bone marrow–derived cells targeted by the Tie2p/e vector, we achieved substantial inhibition of angiogenesis. Future studies will clarify whether TEM cells act directly on angiogenesis within the tumor and whether their involvement is limited to early stages of tumorigenesis.

A small fraction of blood mononuclear cells also expressed the Tie2p/e vector and surface features similar to those of the GFP-positive cells in the tumors of Tie2p/e-BMT mice. Preliminary results indicated that these circulating cells contributed to the tumor TEM cell population. However, commitment to the differentiation program signaled by activation of the Tie2p/e and, consequently, conditional ablation in the experiment described above, may occur in the bone marrow, in the blood or within the tumor. The relationship of the circulating Tie2p/e-marked cells with the previously described EC progenitors[24] was not investigated in our study, although the surface features would distinguish them.

In conclusion, our gene-marking and conditional-ablation studies indicated that TEM cells are a suitable vehicle for gene-based therapy directed to tumors. Tie2 receptor expression has been reported in subsets of hematopoietic stem cells[6], thus potentially limiting the choice of therapeutic genes because of concerns of myelotoxicity. Although we did not note myelosuppression when we delivered a conditionally toxic gene, further long-term studies are needed to

address this. For future applications, autologous bone marrow cells or peripheral blood mononuclear cells could be transduced *ex vivo* as described here and could be used to deliver secreted therapeutics at the tumor sites. In addition, TEM cells may represent a new target for the development of anticancer drugs. Our results have provided 'proof of principle' of a new gene therapy paradigm in which *ex vivo* transduction of multipotent progenitor cells could be used to deliver and selectively express genes into individual organs *in vivo*.

## METHODS

**Vectors.** To generate the Tie2p/e-GFP lentiviral vectors, we cloned a 2,085-base-pair fragment from the mouse Tie2 promoter (*Hin*dIII) and a 1,658-base-pair fragment containing the Tie2 intronic enhancer (*Xho*I/*Kpn*I), both from pHHSDKXK[7], into the lentiviral vector pRRLsin.cPPT.hCMV.GFP.Wpre[9] in place of the CMV promoter and in the *Mfe*I site in the intron derived from HIV-1, respectively. We cloned the herpes simplex virus thymidine kinase gene[25] upstream of the encephalomyocarditis virus IRES and GFP, and inserted the bicistronic cassette in place of the GFP sequence in the PGKp and Tie2p/e lentiviral vectors. Concentrated lentiviral vector stocks, pseudotyped by the vesicular stomatitis viral envelope, were produced as described[8] and their titers were determined using human umbilical vein ECs. Expression titers were $5 \times 10^9$ to $2 \times 10^{10}$ transducing units per ml with an HIV-1 p24 concentration of 100–500 μg/ml.

**Mice.** CD1 homozygous nude, C57BL/6, C57BL/6-TgN(ACTbEGFP)1Osb[11] and FVB/N-TgN(TIE2GFP)287Sato[10] mice were purchased from Charles River Laboratories and were maintained in germ-free conditions. All procedures were done according to protocols approved by the Torino University bioethics committee and the Italian Ministry of Health.

**Tumor studies.** After subcutaneous injection of $5 \times 10^5$ to $5 \times 10^6$ mouse mammary carcinoma (TS/A and N202.1A), melanoma (B16/BL) and Lewis lung carcinoma (LLC/3LL) cells into nude (TS/A, N202, LLC, B16) or syngeneic immunocompetent (LLC, B16, N202) mice, tumors were grown for 1–4 weeks. Tumor size was determined by caliper measurements and tumor volume was calculated by a rational ellipse formula ($m_1 \times m_1 \times m_2 \times 0.5236$, where $m_1$ is the shorter axis and $m_2$ is the longer axis). For quantification of tumor vascularization, three sections of each of three tumors per group were immunostained for CD31 and scanned at ×200 magnification by confocal microscopy to identify regions of high vascular density. We counted $CD31^+$ vessels in three fields at ×200 magnification selected from these regions for each section. Counts were averaged to determine the microvessel density (vessels/$mm^2$) for each experimental group. All values are expressed as average ± s.e.m. The differences between experimental groups were analyzed by Student's *t*-test and were considered statistically significant at $P < 0.05$.

**Immunofluorescence and confocal microscopy.** Anesthetized mice were killed by intracardiac perfusion with 0.9% NaCl followed by 4% paraformaldehyde in PBS (pH 7.4). Organs and tumors were fixed for 2–4 h in paraformaldehyde, equilibrated for 48 h in 15% sucrose in PBS and embedded in optimal-cutting-temperature compound for quick freezing. Cryostatic sections 6 μm thick were post-fixed with paraformaldehyde and frozen at –80 °C. Sections were blocked with 5% goat serum (Vector Laboratories) in PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 (PBS-T). When mouse monoclonal antibodies were used, sections were blocked using the M.O.M. kit (Vector Laboratories). The following primary antibodies were diluted 1:200 in PBS-T with 2% goat serum and 1% BSA: IgG2aκ isotype control, clone A110-2; anti-CD31 (PECAM-1), clone MEC 13.3; anti-CD45 (Ly-5), clone 30-F11; anti-Gr-1 (Ly-6G), clone RB6-8C5; anti-Sca-1 (Ly-6A/E), clone E13-161.7; anti-CD34, clone RAM34 (all from BD PharMingen); anti-F4/80, clone A3-1; anti-CD11b (Mac-1), clone MI/70.15 (Serotec); anti–smooth muscle actin, clone 1A4 (Sigma); anti-Tie2/TEK, clone Ab33 (Upstate Biotechnology). The rabbit affinity-purified GFP antibody was from Molecular Probes. After 1 h of incubation, sections were washed and stained for 1 h with secondary antibodies (AlexaFluor488-conjugated goat anti-rabbit antibody, AlexaFluor546-conjugated goat anti-rat antibody and AlexaFluor546-conjugated goat anti-mouse antibody; Molecular Probes) in PBS-T and 1% BSA. Cell nuclei were stained using TO-PRO-3 (Molecular Probes). For triple labeling, primary rat antibodies conjugated to R-phycoertyithrin or R-phycoertyithrin-Cy5 were used. Confocal microscopy used a three-laser confocal microscope (Radiance 2100; BioRad). Fluorescent signals from single optical sections were sequentially acquired and analyzed by Paint Shop Pro 7.02 (JascSoftware).

**Bone marrow transplantation.** Six-week-old male mice were killed with $CO_2$ and their bone marrow was collected by flushing femurs and tibias. Lineage-negative cells were purified with a kit from StemCell Technologies. For Southern blot analyses, $5 \times 10^5$ lineage-negative cells/ml were transduced with increasing doses of vector ($1 \times 10^7$ to $1 \times 10^8$ human umbilical vein endothelial cells transducing units per ml) in serum-free StemSpan medium (StemCell Technologies) without cytokines, and were expanded and lysed for DNA extraction. DNA was digested with *Afl*II and probed for GFP sequence. Plasmid standards were used to calculate the amount of integrated vector. For BMT, cells transduced with the highest vector dose were injected into the tail vein ($1 \times 10^6$ cells/mouse) of 6-week-old female mice lethally irradiated to allow full engraftment of the transplanted hematopoietic stem cells (8 Gy). Blood and marrow samples were analyzed by FACS after red blood cell lysis and propidium iodide staining. For clonogenic assays, we plated $1 \times 10^4$ and $5 \times 10^4$ bone marrow cells in a methylcellulose-based medium (MethoCult M3434; StemCell Technologies). We assigned scores to colonies for GFP fluorescence and lysed them to detect GFP sequences by PCR[9].

**Partial hepatectomy and organ studies.** C57BL/6 and CD1 nude mice were anesthetized with ketamine and xylazine and subjected to midventral laparatomy. We did a one-third hepatectomy by removing the left liver lobe according to the methods of Higgins and Anderson. Animals were killed 7–10 d after hepatectomy. Organs were collected after intracardiac perfusion as described above. For PCR analysis, tissues were collected without fixation, and DNA was extracted and diluted to obtain a nonsaturating amplification of GFP, as described before[9].


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*Note: Supplementary information is available on the Nature Medicine website.*

### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests

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