Using the Transcription Factor Inhibitor of DNA Binding 1 to Selectively Target Endothelial Progenitor Cells Offers Novel Strategies to Inhibit Tumor Angiogenesis and Growth


Abstract

Tumor angiogenesis is essential for malignant growth and metastasis. Bone marrow (BM)–derived endothelial progenitor cells (EPC) contribute to angiogenesis-mediated tumor growth. EPC ablation can reduce tumor growth; however, the lack of a marker that can track EPCs from the BM to tumor neovasculature has impeded progress in understanding the molecular mechanisms underlying EPC biology. Here, we report the use of transgenic mouse and lentiviral models to monitor the BM-derived compartment of the tumor stroma; this approach exploits the selectivity of the transcription factor inhibitor of DNA binding 1 (Id1) for EPCs to track EPCs in the BM, blood, and tumor stroma, as well as mature EPCs. Acute ablation of BM-derived EPCs using Id1-directed delivery of a suicide gene reduced circulating EPCs and yielded significant defects in angiogenesis-mediated tumor growth. Additionally, use of the Id1 proximal promoter to express microRNA-30–based short hairpin RNA inhibited the expression of critical EPC-intrinsic factors, confirming that signaling through vascular endothelial growth factor receptor 2 is required for EPC-mediated tumor biology. By exploiting the selectivity of Id1 gene expression in EPCs, our results establish a strategy to track and target EPCs in vivo, clarifying the significant role that EPCs play in BM-mediated tumor angiogenesis.

Introduction

The tumor microenvironment is composed mainly of bone marrow (BM)–derived cells, which are critical to angiogenesis-mediated tumor growth and spread (1). These include myeloid cells such as Tie-2+ and GR-1+ monocytes (2–5), tumor-associated macrophages (6, 7), inflammatory cells (8, 9), platelet-derived growth factor receptor β+ pericytes (10), and endothelial progenitor cells (EPC; refs. 11–13). Of particular interest are the BM-derived EPCs, as they have been shown to play a significant role in the growth of early tumors and metastatic lesions by mediating the angiogenic switch (14–17). EPCs have also been proposed to provide an alternative source of tumor endothelium, and thus contribute to neovessel formation by directly incorporating into nascent vasculature as differentiated endothelial cells (11–13). Therefore, EPCs represent important new targets for novel antiangiogenic therapies without the side effects associated with current therapies, which also target host vasculature (14–17).

EPCs are detected as a unique cell population in the peripheral blood (PB), expressing a variety of cell surface markers, which identify them as vascular and BM-derived. Key EPC markers include vascular endothelial (VE)-cadherin, vascular endothelial growth factor receptor 2 (VEGFR2), CD31low, c-kit, and prominin 1/AC133 (16–19). However, the expression of these markers differs depending on whether the EPCs are in the BM, PB, or tumor. This lack of a single marker to be able to unambiguously track EPCs has led to several recent cancer studies failing to identify EPCs in specific mouse tumor models (18–21). This has also raised concerns as to whether the same population is being truly monitored in vivo, and has imposed tremendous limitations on the assessment of the biological function of tumor-associated EPCs as well as their potential as targets for antiangiogenic cancer therapy.

Previous studies have shown that, like tumor-activated endothelium, BM-EPCs also express the transcription factor inhibitor of DNA binding 1 (Id1). Id1 is a member of the helix-loop-helix family of transcription factors and a marker...
of self-renewal (22). We have previously shown that global inhibition of Id1 in the BM results in significant specific EPC-linked tumor vascular defects (17, 23). These findings nominate Id1 as a potential marker of EPCs.

The aims of this study were to determine if Id1 could be used to track EPCs in vivo, specifically target EPCs, and modify EPC-mediated tumor growth. Using a knock-in Id1 reporter mouse (24) and lentiviral (LV) transduction of BM transplanted (BMT) into wild-type (WT) recipients, we have shown that the Id1 gene activity can be used to track and modify BM-EPCs in the BM, blood, BM compartment of the tumor stroma, and luminally incorporated BM-EPCs. Furthermore, selective ablation of EPCs in vivo, using the Id1 proximal promoter (pr/p) to drive the expression of the suicide gene herpes simplex virus (HSV)-thymidine kinase (tk), resulted in specific EPC-linked vascular defects and impaired tumor growth. Directed delivery of short hairpin RNA inhibition (shRNAi) by the LV-Id1pr/p was also used to inhibit key EPC-linked factors, such as VEGFR2, resulting in marked EPC and angiogenesis-linked tumor growth defects. This work underscores the functional importance of EPCs in tumor biology and directly links EPC-intrinsic Id1 to EPC biology and EPC-mediated tumor vascular growth. It also shows that EPCs represent a unique lineage that can be tracked from the BM to tumor using Id1.

**Materials and Methods**

**Mice**

WT C57BL/6 mice, C57BL/6 Id1+/GFP transgenic reporter mice (24), and green fluorescent protein (GFP)-transgenic C57BL/6-Tg (ACTb-GFP) 10sh/j mice expressing GFP under a hybrid chicken β-actin promoter and cytomegalovirus intermediate early enhancer (25) were purchased from The Jackson Laboratory. All procedures involving mice were conducted in accordance with protocols reviewed and approved by the institutional animal care and ethics committees.

**Cell lines and growth conditions**

Murine Lewis Lung Carcinoma cells (LLCs)/D122 (provided by Lea Eisenbach, Weizmann Institute of Science, Rehovot, Israel), murine lymphoma cells B6Rv2 (14), and murine endothelial cells (mHEVc; provided by J. Cook-Mills, University of Cincinnati, Cincinnati, OH; ref. 26) were maintained in RPMI with 15% fetal bovine serum. Murine myoblast progenitors C2C12 cells (American Type Culture Collection) and human kidney 293T cells were maintained in DMEM supplemented with 10% FCS.

**Screening shRNAs**

shRNAs targeting Id1 were cloned into a U6 or Id1 promoter–containing vector as described (27). shRNA targeting firefly luciferase served as a nonspecific control. SYBR Green I detection (28) was used for reverse transcription-PCR (RT-PCR) analysis using the ABI7700 real-time PCR detection system (Applied Biosystems). The primers used for RT-PCR analysis were VEGFR2 (forward, 5′-ATCGTGATATCAGCACTGGAGAAAC-3′, and reverse, 5′-GGGATTGATCTGATCATGTAAC-3′) and Id1 (forward, 5′-GTACTTGGTCTGGGACCC-3′, and reverse, 5′-CATGGCTGTAGAACCGAGC-3′). For internal controls, β-actin (forward, 5′-GTATTGAGACCTTCAACC-3′, and reverse, 5′-TAGGAGCAGAGCAGTAACT-3′) and GADPH (forward, 5′-TCAACGACCTTTCAATTGAC-3′, and reverse, 5′-ATGCGGGATGATGTCTGG-3′) were used.

**Generation of LV constructs**

To generate pWPT-Ω, a gateway destination cassette ccdB (Invitrogen) was inserted into the SalI site in the LV pWPT (D. Torono, University of Geneva, Geneva, Switzerland), and shRNAs were transferred from shuttle vectors by gateway recombination. To generate Id1pr/p-GFP LV, a 1.32 kb fragment containing the murine proximal Id1 promoter was amplified from C57BL/6 mouse genomic DNA and inserted into the pWPT-LV, replacing the EF1α short promoter. To create Id1pr/p-red fluorescent protein (RFP), GFP was replaced (MluI/Xhol) with monomeric RFP (mCherry; ref. 29). To generate pWPT-Id1pr/p-GFPPTK, the HSV-tk gene was amplified from pSHTK (provided by Ventura, Universidade de São Paulo, São Paulo, Brazil) and inserted downstream of GFP/RFP and an internal ribosome entry site (provided by Patrick Paddock, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY).

**LV production, BM transduction, and transplantation**

LV pseudotyped with the vesicular stomatitis virus G protein (VSVG) was generated by calcium phosphate transfection into 293T cells with three packaging constructs, pMDLg/pRRE, REV, and pVSVG, as described (30). Viral titer was determined by p24 ELISA (Perkin-Elmer) or fluorescence-activated cell sorting (FACS) analysis of LV-infected 293T cells in the presence of serum. LV transductions of lineage-depleted BM cells (multiplicity of infection of ~ 50) were performed in serum-free StemSpan SFEM medium (Stem Cell Technologies) in the presence of interleukin-3 (IL-3; 20 ng/mL), IL-6 (100 ng/mL), and stem cell factor (100 ng/mL) for 12 hours. For BMT, 5 × 10⁵ lineage-depleted BM cells were injected into the tail veins of lethally irradiated (1,100 rad) C57BL/6 mice. After BMT, RT-PCR analysis was used to determine the transduction efficiency of LV-infected BM cells. DNA was isolated from the BM and subjected to RT-PCR using GFP-specific primers: forward, 5′-GCTCTGCCCCCTCTCATTGACA-3′, and reverse, 5′-GTGAAACAGCTCTCCTCCGCTTT-3′. A standard curve was generated from titrated target DNA, and the vector copy number was determined.

**Analysis of tumors, blood, and BM by immunohistochemistry and microscopy**

C57BL/6 mice were inoculated intradermally with 5 × 10⁶ LLC or B6Rv2 cells, and tumor size was monitored and analyzed by microscopy (16). For the analysis of circulating endothelial progenitors (CEP), tail blood was collected in anticoagulant buffer (PBS, 5 mmol/L EDTA); for the analysis of BM-EPCs, the BM was flushed from the bones. PB...
mononuclear cells and BM mononuclear cells were isolated by gradient centrifugation using Histopaque 1077 (Sigma) and then either cytospun onto Superfrost slides or stained for FACS analysis. Primary antibodies, CD31/platelet/endothelial cell adhesion molecule 1 (clone MEC 13.3), VE-cadherin/CD144 (clone 11D4.1), CD11b (clone M1/70), VEGFR2/Fk1 (clone avas12e1), GR-1 (clone RB6-8C5), pan CD45 (clone 500A2), B220 (clone RA3-6B2), CD3 (clone 145-2C11), c-kit/CD117 (clone 2B8), CD11b (clone M1/70), and TER-119 (clone TER-119), were obtained from BD Pharmingen; prominin 1 (clone 13A4) was from eBiosciences; and Ki-67 (clone SP6) was from NeoMarkers. Unless otherwise stated, EPCs/CEPs were selected as VE-cadherin+, VEGFR2+, c-kit+, CD45−, and CD11b− subpopulations isolated by FACS from the mononuclear population in the BM/blood. Myeloid populations were selected as CD11b+ (myeloid cells) and GR-1+ (neutrophils), Tie-2+ (Tie-2+ monocytes), B220+ (B cells), or CD3+ (T cells), and isolated by FACS from the mononuclear population in the blood or BM. All populations were negative for TER119 (erythroid marker).

Microscopy was performed using 30-μm-thick sections, stained with Alexa Fluor–conjugated primary antibodies.
with 4′,6-diamidino-2-phenylindole staining for cell nucleus (Invitrogen). In some cases, Alexa Fluor–conjugated secondary antirabbit antibodies were used for staining with anti-Id1 monoclonal antibody (22). RFP/GFP+ cells were detected by their own signal. Fluorescent images were obtained using a Zeiss fluorescent microscope (Software Axiovision LE 4) with resolutions of 0.275 to 0.35 μm as described (16).

**FACS analysis**

Single-cell suspensions were preblocked with Fc block (CD16/CD32, BD Pharmingen) and incubated with primary antibodies; these include IgG2aκ and IgG2aβ isotype controls and various antibodies described previously. Labeled cells were measured by a LSRII flow cytometer (Becton Dickinson), and compensation was measured using FACSDiva software (BD Immunocytometry Systems). Animals were also injected with Alexa Fluor 647–conjugated isoelectin GS-IB4 (50 μg for 10 minutes; Molecular Probes) before sacrifice. Multivariate FACS was performed using isotype antibodies, fluorescence-minus-one samples, and unstained samples for determining appropriate gates, voltages, and compensation (31).

**Statistics and data analysis**

Statistical analysis was performed using GraphPad Prism software (version 3.0). Statistical analysis of tumor growth by one-way ANOVA (α = 0.05) was used to compare different treatments. For comparison of groups at end point, Student’s t test (α = 0.05) was used. Unless otherwise stated, data are presented as mean ± SEM.

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**Figure 2.** Id1 marks luminally incorporated, mature EPCs in the nascent tumor vasculature. A, top, microscopic images showing vasculature and BM-derived Id1+GFP− CD31+low CD11b− BM-EPCs as part of the tumor stroma (arrows) of LLC tumors (day 15) implanted into Id1+/GFP− BMT mice (n = 10). Bar, 50 μm. Bottom, microscopic images of a tumor vessel showing an incorporated BM-derived mature EPC (Id1−GFP+ CD31+ CD11b−; arrow) in LLC tumors (day 15) implanted into Id1−/GFP− BMT mice. Bar, 20 μm. B, high-resolution (×63) transverse immunofluorescent images of a tumor vessel showing an incorporated BM-derived mature EPC (Id1−GFP+ CD31+ CD11b−; arrow) in LLC tumors (day 15) implanted into Id1−/GFP− BMT mice. Bar, 20 μm. C, summary of FACS analysis showing the contribution of Id1+ mature EPCs in functional vessels by isoelectin staining in LLC tumor (day 6) in Id1pr/p-GFP BMT mice. D, representative scatter plots showing derivation of GFP gates. SSCA, side scatter values.
Results

Id1 is upregulated in the BM and tumor-associated EPCs

Gene expression profiling showed that Id1 was one of several genes significantly upregulated at least 4-fold in BM stem cells obtained from tumor-challenged mice (Supplementary Fig. S1A). As Id1-knockout mice exhibit impaired EPC-linked angiogenic and tumor growth defects (14, 19), we next determined to localize Id1 to specific BM-derived cell populations in the tumor stroma. To do this, we transplanted the BM from β-actin (ACTb)-EGFP mice (25) into irradiated age-matched, syngeneic, WT recipients and examined preangiogenic, as well as vascularized, tumors after intradermal inoculation of LLC cells. Notably, Id1 protein was confined to BM-derived GFP+ VE-cadherin+ EPCs (16) and not to other BM-derived GFP+ myeloid cells (Supplementary Fig. S1B).

The Id1 gene activity marks EPCs in the BM, blood, and tumor stroma

To determine whether a genetic strategy using the Id1 gene activity might be used to track EPCs and investigate EPC function in vivo, the pr/p of Id1 (1.32 kb), containing...
known Id1 regulatory sequences (32) and driving fluorescent reporter genes (GFP and RFP/mCherry), were cloned into the pWPT, a self-inactivating LV vector (ref. 29; Fig. 1A and B, top). The functionality of the Id1pr/p constructs was confirmed after stable transduction. In this experiment, dose-dependent enhancement of reporter activity was observed after administration of serum (not shown) or growth factors to cell lines stably transduced with the LV-Id1pr/p constructs (Supplementary Fig. S2A).

To evaluate the selectivity of Id1pr/p for EPCs in vivo, BM cells not yet expressing key lineage markers (lineage-depleted BM; see Materials and Methods), obtained from WT mice, were transduced with Id1pr/p-GFP ex vivo and transplanted into irradiated WT recipients. To determine the relative abundance of EPCs with respect to other BM-derived cells, lineage-depleted BM cells derived from Actb-EGFP<sup>+</sup> mice were also transduced with LV-Id1pr/p-RFP. After BM engraftment, RT-PCR analysis showed an average of two to four LV integrations per cell (Supplementary Fig. S2B). Id1pr/p-GFP/WT and Id1pr/p-RFP/Actb-EGFP<sup>+</sup> BMT animals were then LLC tumor challenged. Analysis of early tumors (days 6–8) showed recruitment of Id1pr/p-GFP<sup>+</sup> VE-cadherin<sup>+</sup> cells to the tumor periphery (Fig. 1A and B, bottom). In addition to VE-cadherin, these cells also expressed other EPC markers (16), including VEGFR2 and prominin 1 (Supplementary Figs. S3A and B). Furthermore, none of these Id1pr/p<sup>+</sup> cells expressed the myeloid marker CD11b<sup>+</sup>, thus indicating that these cells are EPCs (Supplementary Fig. S3C). In the blood, reporter analysis showed that the majority of VEGFR2<sup>+</sup> c-kit<sup>+</sup> CD11b<sup>+</sup> CEPs (80%; <i>P < 0.001</i>) were Id1pr/p<sup>+</sup>, whereas BM hematopoietic cells of the myeloid lineage expressed negligible levels of Id1pr/p activity (0.09%; <i>P < 0.001</i>; Supplementary Fig. S4A and B; Supplementary Table S1). Additionally, analysis of the BM showed that Id1pr/p activity was restricted to VE-cadherin<sup>+</sup> CD11b<sup>+</sup> CEPs (66.83%; <i>P < 0.001</i>), thus indicating that the Id1pr/p are marking EPCs (Supplementary Fig. S4C; Supplementary Table S1). Finally, the Id1pr/p<sup>+</sup> cells also expressed endogenous Id1 protein in the blood, BM, and tumor stroma (Fig. 1C).

To confirm that we had captured the important regulatory regions in the LV constructs used and to determine whether the observed effects were due to positional effects or chromosomal positioning of the LV vector, we took advantage of the recently available Id1<sup>−/−</sup>GFP knock-in fluorescent reporter mice (24). In this experiment, the BM from these mice was transplanted into irradiated WT mice, and tissues were examined in the context of LLC tumor challenge. In confirmation of the LV work, GFP activity was exclusively restricted to VE-cadherin<sup>+</sup> CD31<sup>hi</sup> CD11b<sup>−</sup> BM-derived cells (EPCs) in the tumor stroma of early tumors [Figs. 1D and 2A (top)]

**The Id1 gene activity marks mature EPCs incorporated as part of the functional tumor vasculature**

Analysis of later tumors (days 8–12) from LV and Id1<sup>−/−</sup>GFP<sup>+</sup> BMT mice revealed luminally incorporated GFP<sup>+</sup> CD31<sup>+</sup> CD11b<sup>−</sup> mature EPCs in a subset of tumor neovessels [Fig. 2A (bottom) and B; Supplementary Fig. S5A and B]. This shows that the Id1pr/p also marks mature EPCs incorporated as endothelial cells (16). We next quantified these luminally incorporated BM-derived Id1<sup>+</sup> endothelial cells by FACS after administration of isoleucin GS-B4 (16). Of the total functional vasculature, as determined by lectin<sup>+</sup> CD31<sup>+</sup> CD11b<sup>−</sup> cells, 9.4 ± 2.6% of the vessel-incorporated luminal endothelial cells (GFP<sup>+</sup> lectin<sup>+</sup> CD31<sup>+</sup> CD11b<sup>−</sup>) at day 8 were BM-derived and expressed the Id1pr/p (Fig. 2C and D).

**Ablation of Id1<sup>+</sup> cells results in angiogenesis inhibition and impaired tumor growth**

To determine the biological function of EPCs in tumor angiogenesis and to accomplish selective EPC ablation, the Id1pr/p-LV was used to express the suicide gene HSV-<i>tk</i> (ref. 33; Fig. 3A, top; Supplementary Fig. S6A–C). This was done by transducing lineage-depleted BM cells with the LV-Id1pr/p-GFPITK and transplanting these cells into irradiated WT recipients. The administration of ganciclovir (GCV) showed a significant delay in tumor growth in Id1pr/p-GFPITK mice (70%; by day 20) compared with controls (Fig. 3A, bottom).

FACS analysis showed that GCV treatment resulted in a 3-fold reduction in CEPs in the PB of Id1pr/p-GFPITK mice (Fig. 3B). As there was no significant change in other BM-derived hematopoietic cells or progenitors, it can be concluded that this reduction was CEP specific (Fig. 3B). We next sought to determine whether this reduction in CEPs was due to <i>tk</i>-mediated BM-EPC ablation. Analysis of the BM showed a 2-fold reduction in EPCs, with no significant reduction in CD11b<sup>−</sup> hematopoietic cells in Id1pr/p-GFPITK (+GCV) mice (Fig. 3C). In concordance with this observation, actively proliferating EPCs were observed in tumor-challenged BM, as judged by Ki-67 staining (ref. 34; Supplementary Fig. S6D). The Id1pr/p-GFPITK (+GCV) tumors also showed a significant reduction in vessel density and growth compared with untreated Id1pr/p-GFPITK controls (Fig. 3D; <i>P < 0.0001</i>). These results show that Id1pr/p-mediated delivery of tk, while resulting in the selective elimination of EPCs, seemed to also be associated with limited bystander effects, a complicating factor in many GCV/<i>tk</i>-based approaches (35).

**Id1-directed silencing of EPC-intrinsic factors results in inhibition of angiogenesis-mediated tumor growth**

To determine whether EPC-intrinsic factors, such as Id1 and VEGFR2, were required for EPC-mediated tumor angiogenesis and whether Id1 ablation specifically affected EPC biology in the context of tumor challenge, we used short hairpin (Ω) RNAi, delivered by the LV-Id1pr/p construct, to directly inhibit BM-EPCs. First, to assess LV-mediated shRNA delivery <i>in vivo</i>, vectors with the constitutive U6 promoter driving RNAi were used (Supplementary Fig. S7A and B, top). As expected from the EPC-linked tumor angiogenesis defects observed in Id1-knockout mice (14), BM-wide suppression of Id1 resulted in impaired B6R2V lymphoma (Supplementary Fig. S7B, bottom right) and LLC (Supplementary Fig. S7B, bottom left) tumor growth. This finding was associated with reduced tumor vascularization and Id1 mRNA suppression (Supplementary Fig. S7C and D).

To facilitate expression by the poll1 Id1 promoter, miR-30–based shRNAs (36) were then cloned into LV-Id1pr/p (Fig. 4A,
The effectiveness of the LV-Id1pr/p constructs to drive each shRNAi, after stable integration, was determined using fluorescence and quantitative RT-PCR (Supplementary Fig. S8A and B). Next, WT lineage-depleted BM was transduced with Id1pr/p-GFPNSΩ, Id1pr/p-GFPlid1Ω, and Id1pr/p-GFPVEGFR2Ω LV constructs, respectively, and transplanted into irradiated WT mice. Tumor growth in Id1pr/p-GFP Id1Ω and VEGFR2Ω BMT animals was impaired compared with nonspecific control [Fig. 4A (bottom) and B]. Notably, Id1pr/p shRNA–mediated suppression of both Id1 and VEGFR2 resulted in a 2- to 3-fold reduction of CEPs (Fig. 4C; Supplementary Fig. S9A), but no significant change in CD45+ c-kit+ hematopoietic cells (Fig. 4C; Supplementary Fig. S9B and C), GR-1+ neutrophils (Supplementary Fig. S9D), Tie2+ monocytes, B220+ B cells, CD3+ T cells, or CD11b+ c-kit+ myeloid progenitors (Supplementary Fig. S10A–D), thus indicating that the effect of this suppression is CEP specific. Analysis of tumors also showed a significant reduction in vessel density in Id1pr/p-GFPlid1Ω and Id1pr/p-GFPVEGFR2Ω BMT mice compared with control mice (Fig. 4D). RT-PCR analysis of the BM confirmed that Id1 or VEGFR2 shRNAs had indeed suppressed cognate target genes in vivo. A 60% reduction in Id1 mRNA was observed in Id1Ω BM, whereas a 75% reduction was observed in VEGFR2 mRNA in VEGFR2Ω BM compared with NSΩ BM. The levels of pan hematopoietic CD45 mRNA also remained unchanged (Supplementary Fig. S8C). This experiment was not influenced by differential LV transduction efficiency or shRNA-mediated preferential enrichment of specific populations, as the BM of all Id1pr/p-GFP transduced animals showed comparable integrations per cell (Supplementary Fig. S8D). Taken together, these results show that the EPC-intrinsic expression of Id1 and VEGFR2 is critical for effective EPC mobilization from the BM and for normal tumor vascularization and growth.

Discussion

Whereas the contribution of BM-derived EPCs to tumor neovessel formation has been reported in mice and humans (12, 16, 37, 38), the inability to deliver transgenes specifically...
to EPCs in vivo has precluded the analysis of their biological function and the assessment of their therapeutic potential. In this study, we show for the first time, using LV Id1 reporter constructs and Id1+/GFP fluorescent reporter mice (24), that the Id1 gene is selective for EPCs and can be used to track EPCs in the BM, blood, and tumor stroma and EPCs incorporated in the tumor vasculature. This identification of luminally incorporated mature EPCs in the tumor vasculature expressing the Id1 gene also validates that Id1 marks cells that are true EPCs. Furthermore, this finding validates that EPCs incorporate into the tumor vasculature. To the best of our knowledge, this study provides the first direct in vivo evidence of EPCs being marked by a single unique marker in each of these tissues.

To confirm the selectivity of Id1 for BM-EPCs, the LV-Id1pr/p construct was used to deliver the suicide transgene HSV-4k. After administration of GCV, EPCs were specifically ablated, resulting in a 60% to 70% reduction in CEPs, as well as angiogenesis inhibition and impaired tumor growth. This Id1-directed specific EPC ablation also did not significantly affect hematopoiesis or other hematopoietic cell populations. These findings are consistent with the observations in Id1-knockout mice and their WT littermates, which showed no difference in hematopoietic progenitors (HPC; ref. 39). Furthermore, although recent studies have shown that continuous serial transplantation of Id1-knockout BM results (ultimately) in impaired engraftment potential [due to a reduction in long-term repopulating hematopoietic stem cells (HSC)], Id1 was found to be dispensable for the short-term recovery of HSCs (24, 40). This is supported by the observation in this study that acute suppression of Id1 in the adult BM compartment, although resulting in tumor angiogenic defects, was not associated with HSC defects in our HSV-tk/GCV–treated animals.

The LV-Id1pr/p construct was also designed to express shRNAs designed after an endogenous miRNA and determine the function of the endogenous EPC-specific genes Id1 and VEGFR2 in tumor angiogenesis. shRNA-mediated suppression of Id1 resulted in EPC mobilization defects (70–80% reduction in CEPs) associated with severe angiogenesis inhibition and impaired tumor growth, with no significant change in cells of the hematopoietic lineage (B cells, T cells, CD11b+ myeloid cells, and Tie2+ monocytes). This result is consistent with the observations made following the HSV-tk delivery in Id1 transgenic mice (14, 24). However, our result differs slightly from that of Jankovic and colleagues (40), who reported reduced number of circulating lymphocytes in the PB of resting Id1-knockout mice. Possibly, in our study, acute and short-term suppression of Id1 in the BM compartment is devoid of the developmental compensations in the hematopoietic system associated with the Id1-knockout animal. In another study, Lyden and colleagues (14) showed that angiogenesis inhibition in the Id1 mutant was due to defects in mobilization of both VEGFR2+ EPCs and VEGFR1+ CD11b+ hematopoietic cells. However, the use of the combined Id1−/−Id3null genotype in their study may have resulted in defects in VEGFR1+ cell mobilization as a result of Id3 loss, as described (41). Furthermore, we have observed that Id1 silencing (either by shRNAi or in Id1-knockout mice) specifically affects VEGFR2+ EPCs and not VEGFR1+ cells (20).

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