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SUPPLEMENTAL DATA

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Supplemental data for:

Shaked et al., *Cancer Cell* 7, pp. 101–111

Supplemental experimental procedures**Animal models and strains**

All animal work was done in accordance to institutional guidelines (Sunnybrook and Women's College Health Sciences Centre, Toronto, and the European Institute of Oncology, Milan). The analyses of all strains were performed on groups of four to six males, 9–12 weeks old, unless indicated otherwise. C57BL/6J, 129/SvImJ, and BALB/cJ mice were purchased from Jackson Laboratories, Bar Harbor, ME. CD-1 mice were purchased from Charles River Canada. DBA/2J, CBA/J, C3H/HeJ, and FVB/NJ mice were purchased from Charles River, Italy. Mice were first anesthetized and bled by cardiac puncture followed by cervical dislocation. Blood was stored in EDTA tubes on ice, prior to the assessment of circulating endothelial cells (CECs)/circulating endothelial progenitor cell (CEPs).

The generation of homozygous TSP-1 null mice on a C57BL/6J background obtained by backcrossing TSP-1 heterozygous mice eight times to wild-type C57BL/6J mice, as described elsewhere (Lawler et al., 1998). The generation of VEGF hypermorphic mice (VEGF-A^{hi/+}) which express moderate increases of VEGF-A levels were obtained by insertion of lacZ gene followed by an SV40 polyadenylation signal between the stop codon and the 3'UTR of the VEGF-A gene, as described in detail elsewhere (Miquerol et al., 2000). The generation of Tie-2 overexpression mice obtained by construction and expression of the driver ptektTA and responder pte^{OS}tek transgenes as described previously (Sarao and Dumont, 1998; Jones et al., 2001). Briefly, the driver line was generated by injecting a transgene containing a minimal *tek* promoter driving the expression of the tetracycline transactivator tTA, while the responder line was generated by injecting a transgene containing the tetracycline DNA binding operator sequences upstream of the *tek* cDNA.

Corneal neovascular micropocket assay

The corneal neovascular micropocket assay was performed as described (Kenyon et al., 1996) using pellets containing 180 ng carrier-free human recombinant VEGF 165 (R&D Systems, Minneapolis, MN). The area of vascular response was assessed on the sixth postoperative day using a slit lamp. Vessel area was calculated as was previously described (Kenyon et al., 1997) using the equation $0.2\pi \times VL \times CH$, where VL is vessel length from the limbus in mm and CH is clock hours around the cornea. At least five male mice, 8 weeks old or older (ten eyes per strain) were analyzed, and similar numbers of control C57BL/6J animals were included in each assay to confirm consistency. Mouse strains for this method were obtained from Jackson Laboratories (Bar Harbor, ME) or Charles River Labs (Wilmington, MA) and were housed in Children's Hospital's animal facility (Boston) on standard diet and bedding until the assay was performed. Eye assays were conducted according to protocols approved by the Institutional Animal Care and Use Committee of Children's Hospital.

In vivo angiogenesis assessment by Matrigel plug perfusion assay

The Matrigel plug perfusion (angiogenesis) assay was performed as previously described (Klement et al., 2000; Bocci et al., 2003) with some minor modifications. Briefly, 0.5 ml Matrigel (Collaborative Biomedical Products) supplemented with 500 ng/ml bFGF was injected subcutaneously into both flanks of three 10-week-old male mice from each strain, i.e., C57BL/6J, BALB/cJ, and 129/SvImJ. As controls, three 10-week-old male mice of the same strain were injected with Matrigel alone. At day 10, all mice were injected i.v. with 0.2 ml of 25 mg/ml FITC-dextran (Sigma Chemical). Plasma samples were collected, and Matrigel plugs were incubated at 37°C overnight with Dispase (Collaborative Research) and then homogenized. Fluorescence readings were obtained using a FL600 Fluorescence Plate Reader (Biotech Instruments), and angiogenic response was expressed as a ratio of Matrigel plug fluorescence/plasma fluorescence.

CEC and CEP measurements by flow cytometry

Evaluation of CECs and CEPs was carried out on blood collected by cardiac puncture in *VACUTAINER* EDTA tubes (BD *VACUTAINER* systems), followed by enumeration using four-color flow cytometry as described previously (Bertolini et al., 2003). In this regard, we do not recommend using heparinized tubes due to a possible change in the position of peripheral blood cell populations detected by flow cytometry. All fluorochrome-labeled antibodies were purchased from BD Biosciences, Canada. Monoclonal antibodies specific for CD45 were used to exclude CD45⁺ hematopoietic cells, and CECs and their CEP subset were depicted as described previously (Capillo et al., 2003; Monestiroli et al., 2001) using the murine endothelial markers fetal liver kinase 1/VEGF receptor 2 (flk-1/VEGF-R2), CD13, and CD117 (BD Pharmingen, San Diego, CA). Nuclear staining (Procount, BD, San Jose, CA) was conducted to exclude the possibility of platelets or cellular debris interfering with the accuracy of CEC and CEP enumeration (Capillo et al., 2003; Mancuso et al., 2001). After red cell lysis, cell suspensions were evaluated by a FACSCalibur cell analyzer and Cellquest Pro acquisition and analysis program (BD) using analysis gates designed to exclude dead cells, platelets, and debris. Acquisition of at least 100,000 events per sample were obtained in order to analyze the percentage of CECs/CEPs. The absolute number of CECs/CEPs was then calculated as the percentage of the events that were collected in the CEC and CEP enumeration gates, multiplied by the total white count. Percentages of stained cells were determined and compared to appropriate negative controls. Positive staining was defined as being greater than nonspecific background staining, and 7-Aminoactinomycin D (7AAD) was used to enumerate viable versus apoptotic and dead cells, in some experiments (Philpott et al., 1996).

In vivo tumor induction and measurement

Spontaneous erythroleukemia model: viral lysates of the replication competent NB-tropic Friend murine leukemia virus (F-MuLV) were prepared through repeated culturing of the fibroblastic, Clone-B cell line in minimum essential medium- α medium (MEM) (Gibco) supplemented with 10% fetal bovine serum (Gibco) and penicillin/streptomycin at 1000 U/ml (Gibco) as previously described (Shibuya and Mak, 1983). Age-matched BALB/cJ neonates were randomly divided into two groups, infected and noninfected controls, with infections carried out by i.p. administration of 100 μ l of F-MuLV viral lysate via a 1 cc U-100 Insulin Syringe (Becton Dickinson) 1 day post birth. Weaning of all offspring took place 3 weeks post birth, after which they were randomly separated into treated and untreated groups. Tumor were measured by hematocrit levels.

Syngeneic Lewis-Lung Carcinoma (LL/2) cells (0.5×10^6 per 0.2 ml) were subcutaneously injected into the flanks of 10-week-old C57BL/6J mice as previously described (Bocci et al., 2003) and human breast cancer (MDA-MB-231) cells (2×10^6 per 0.05 ml) were injected into the mammary fat pad of 8-week-old CB-17 SCID mice (Charles River, St.-Constant, Quebec, Canada) (Bocci et al., 2004). Tumors were measured by calipers, and the formula $\text{width}^2 \times \text{length} \times 0.5$ was applied for approximating the tumor volume. When tumors had grown to approximately 200 mm³, the mice were randomized into groups of five animals. Then, mice were treated with either DC101 or ABT-510 as indicated in the text.

Human Burkitt's lymphoma (Namalwa) cells (10×10^6 /mouse) were injected i.p. to 6- to 8-week-old CB-17 NOD/SCID mice as previously described (Bertolini et al., 2003). Tumors were measured by calipers as indicated above. Mice were randomized into groups of six animals and treated with DC101.

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Supplemental Table S1. WBC count and percentage of CEC/CEP in different strains and transgenic mice

Strain	WBC	% CEC	% CEP (from CEC)
129/SvImJ	8660 ± 1892	0.182 ± 0.090	32 ± 19
BALB/cJ	5120 ± 766	0.134 ± 0.063	36 ± 11
DBA/2J	12100 ± 4200	0.061 ± 0.011	41 ± 28
CBA/J	14700 ± 3300	0.022 ± 0.008	43 ± 22
FVB/NJ	13800 ± 4100	0.014 ± 0.003	30 ± 16
C57BL/6J	7480 ± 1538	0.046 ± 0.025	29 ± 16
C3H/HeJ	3600 ± 1700	0.025 ± 0.009	11 ± 8
CD-1	3825 ± 330	0.020 ± 0.008	21 ± 7
<i>TSP-1</i> ^{-/-}	7233 ± 2150	0.200 ± 0.080	26 ± 15
<i>VEGF</i> ^{hi/+}	4633 ± 152	0.140 ± 0.020	53 ± 16
Tie-2 overexpression	8700 ± 1612	0.090 ± 0.030	77 ± 12

The WBC count and percentage of CEC or CEP's subset (from the CEC population) are presented in mean ± SD. The strains are ranked from highest to lowest of angiogenic response as well as levels of CEC/CEP, except for the transgenic mice, which are presented below.

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