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Supplemental Data

Article

Rapid Chemotherapy-Induced Acute Endothelial Progenitor Cell Mobilization: Implications for Antiangiogenic Drugs as Chemosensitizing Agents

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Supplemental Experimental Procedures

Chemotherapy Drug Manufacturers

The following chemotherapy drugs used in this study were purchased from the institutional pharmacy: paclitaxel (Apotex inc, Toronto, Ontario, Canada), docetaxel (Sanofi Aventis, Laval, Quebec, Canada), vinblastine (Mayne Pharma Inc, Montreal, Quebec, Canada), gemcitabine (Eli Lilly, Toronto, Ontario, Canada), 5-FU (Mayne Pharma Inc, Montreal, Quebec, Canada), cisplatin (Faulding Canada Inc, Montreal, Quebec, Canada), cyclophosphamide (Baxter Oncology GmbH, Mississauga, Ontario, Canada), irinotecan (Mayne Pharma Inc, Montreal, Quebec, Canada), and doxorubicin (Pharmacia Canada Inc, Mississauga, Ontario, Canada).

Analysis of CEPs by Flow Cytometry

For preclinical evaluation of viable CEPs, blood was obtained from anaesthetized mice via retro-orbital sinus bleeding and prepared for CEP labeling using, as previously described (Bertolini et al., 2003; Shaked et al., 2005). For clinical samples, CEPs were evaluated in patients as described (Bertolini et al., 2006). In brief, few minutes before and 4 hr after chemotherapy, blood was collected in EDTA tubes. Cell suspensions were evaluated after red cell lysis and labeling by flow cytometry, and acquisition of at least 10^5 (in mice) and 10^6 (in patients) events per sample were performed. Analyses were considered informative when adequate numbers of events (ie >50, typically 300-400) were collected in the CEP enumeration gates. At the European Institute of Oncology (Milan, Italy) CEPs were evaluated using FACSCanto and were defined as DNA (Syto16)+/CD45-/CD34+/VEGFR2+ (Case et al., 2007) or DNA(Syto16)+/CD45-/CD133+/VEGFR2+ (Peichev et al., 2000). The kinetics of the two CEP populations did not significantly differ (data not shown). Figure S6 represents a flow cytometry plot of CEP analysis in patients. At the Department of Medical Oncology, University Medical Center (Utrecht, the Netherlands), CEPs were evaluated after isolation of mononuclear cell fractions that were subsequently stored at -70°C until analysis (Norden-Zfoni et al., 2007). Samples were evaluated using FACSCalibur II, and were defined as CD45-/CD31+/CD146+-/CD133+ (Bertolini et al., 2006). In mice, CEPs were evaluated by FACS-LSRII or FACSCalibur II and were defined as

CD45-/CD13+/VEGFR2+/CD117+ (Bertolini et al., 2006). 7-aminoactinomycin D (7AAD) was used to distinguish apoptotic and dead cells from viable cells (Bertolini et al., 2006).

Quantitation and Visualization of Tissue necrosis, Hypoxia, Vessel Perfusion, Tumor Cell Proliferation, and Apoptosis

Tissue processing and immunohistochemistry were performed as described previously (Shaked et al., 2006). Briefly, formalin-fixed, paraffin-embedded tumor sections (4 – 6 μm thick) were stained with hematoxylin and eosin (H&E). Necrotic tissue autofluorescence was detected in the fluorescein isothiocyanate (FITC) channel as previously described (Dragowska et al., 2004). Tumor cryosections (4 – 6 μm) were used for analysis of perfusion by use of the DNA-binding dye, Hoechst 33342 (40 mg/kg) (Sigma-Aldrich Canada Ltd., Oakville, ON Canada) and hypoxia by pimonidazole hydrochloride (60 mg/kg) (Natural Pharmacia International Inc., Burlington, MA) as previously described (Shaked et al., 2006). Hypoxia immunostaining was carried out using the anti-pimonidazole antibody Hypoxyprobe-1 (1:200, Natural Pharmacia International Inc.) and its secondary FITC-conjugated rabbit anti-mouse (1:200, Jackson ImmunoResearch Laboratories Inc). In some experiments, vessels were immunostained with an anti-CD31 antibody (1:200, BD Biosciences, San Diego, CA) and its secondary Cy3-conjugated donkey anti-rat (1:200, Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Proliferating cells were immunostained with a rabbit polyclonal Ki-67 antibody (Vector Laboratories Inc., Burlington, ON, Canada), and its secondary Texas-Red conjugated goat anti-rabbit (1:200, Jackson ImmunoResearch Laboratories Inc) (Shaked et al., 2006). Apoptotic cells were detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) (Roche Diagnostics, Indianapolis, IN). Controls were immunostained with a secondary antibody alone.

Isolation of Platelets and Analysis of SDF-1α

Experiments were performed as previously described (Jin et al., 2006). Briefly, blood was drawn from either treated or untreated C57BL/6 mice by cardiac puncture and collected into sodium citrate tubes (BD Biosciences). To obtain platelet-rich plasma, 750μl blood was mixed with 450μl saline and subsequently centrifuged at 1000 rpm at RT for 5 min. For in-vitro studies, supernatants containing platelet-rich plasma were incubated with paclitaxel or gemcitabine for 4 hours at RT, as described in the text. Platelets incubated with 5ng thrombin were used as a positive control (Sigma-Aldrich Canada Ltd). To measure SDF-1α content in platelets, platelet-rich plasma was centrifuge at 2500 rpm for 5 min at RT. Pellets containing platelets were collected and cell lyses was performed. Samples were normalized according to their protein content measured by Bradford technique using 2% Bis Solution (BioRad Inc., Mississauga, ON , Canada), and subsequently subjected to a specific SDF-1α ELISA (R&D systems) followed by the manufacture's instruction.

Bone Marrow Transplantation

Experimental procedures were carried out as previously described (Shaked et al., 2006). Briefly, GFP+ bone marrow cells (10^7) isolated from femurs of UBI-GFP/Bl6 mice (The Jackson laboratory) were injected into the tail veins of 6-8 week old lethally irradiated (950 rad) C57BL/6 mice. After 3-4 weeks, recipient mice were bled from the orbital sinus to evaluate bone marrow transplantation efficiency using flow cytometry. Those that had >97% GFP+ peripheral blood cells were subsequently used as recipients for injection of LLCs.

Supplemental References

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Table S1. Chemotherapy Drugs Administered near or at the MTD

Class	Drug	Dose (mg/kg)	Reference
Tubulin-binding agents (taxanes)	paclitaxel	30-50	Sharma and Straubinger, 1994; Vanhoefer et al., 1997
	docetaxel	40	Vanhoefer et al., 1997
Tubulin-binding agents (vinca alkaloids)	vinblastine	11	Inaba et al., 1989
Antimetabolites	gemcitabine	120-500	Braakhuis et al., 1991; Chaplin and Hill, 2002; Merriman et al., 1996
	5-FU	100	Saif and von Borstel, 2006
Alkylating Agents	cisplatin	6	Rottenberg et al., 2007
	cyclophosphamide	250	Masnaya et al., 2003
Topoisomerase I inhibitors	irinotecan	100	Azrak et al., 2007
Anthracycline derivates	doxorubicin	12	Inaba et al., 1989

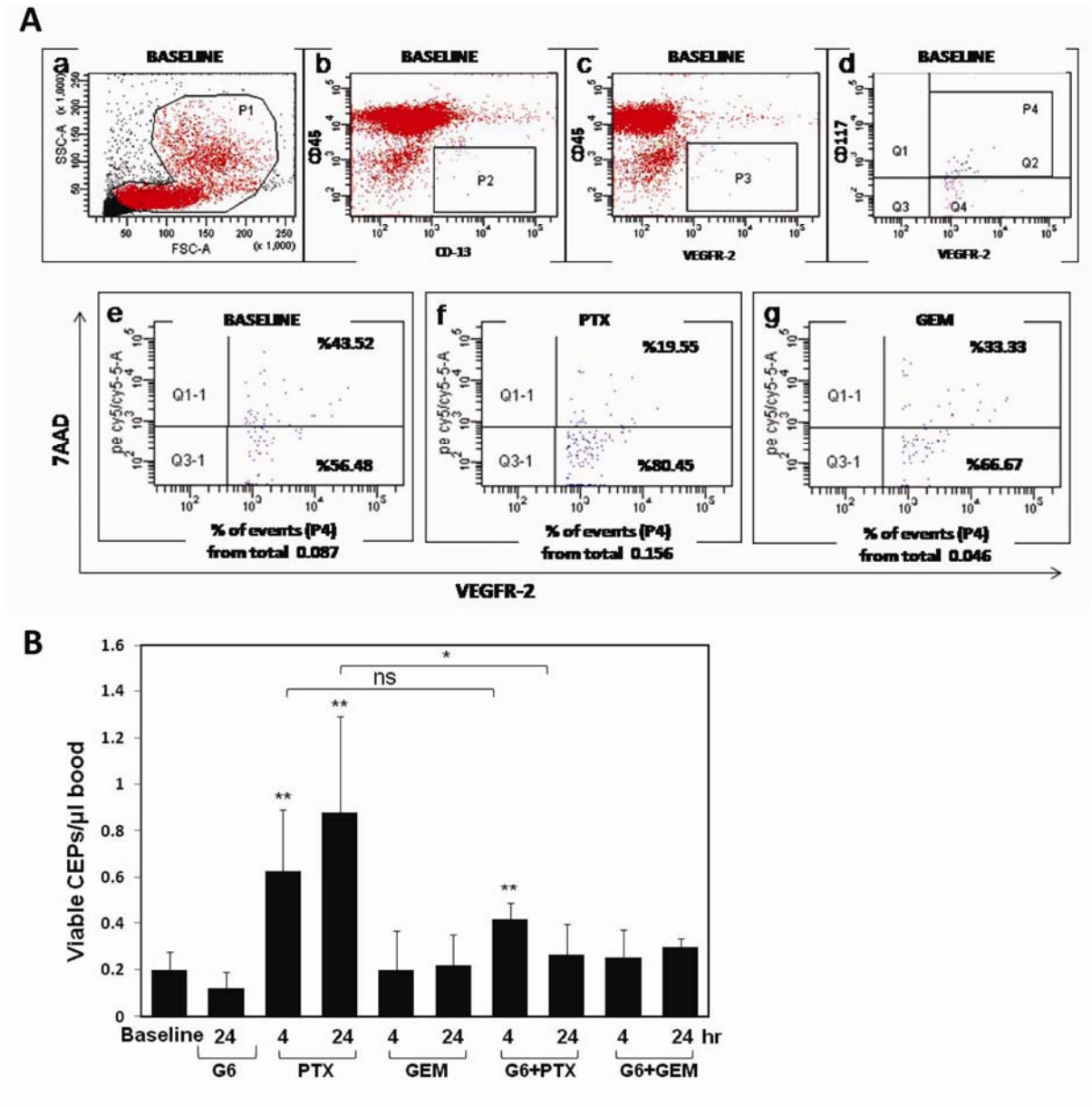

Figure S1. Evaluation of CEPs in Mice Treated with Either Paclitaxel or Gemcitabine in Combination with G6-31

(A) Representative viable CEP enumeration by flow cytometry of non-tumor bearing C57BL/6 mice, 4 hours after they were treated with either 50 mg/kg paclitaxel (PTX) or 500mg/kg gemcitabine (GEM). Panel (a) shows the distribution of peripheral blood cells using forward scattered. (b and c) shows the localization of CD45 positive cells and the subsequent gating of circulating CD45-, and CD13+ or VEGFR2+ cells. Panel (d) presents the position of events from gate p3 on CD117, and subsequent gating (p4) positive to CD117. Panels (e-g) show the cell viability (7AAD- negative) from gate p4.

(B) Non-tumor bearing C57BL/6 mice were treated with 50mg/kg paclitaxel (PTX),or 500mg/kg gemcitabine (GEM) with or without G6-31 antibodies administered 24 hours prior to chemotherapy treatment. Blood was drawn via retro-orbital sinus for the evaluation of viable CEPs using flow cytometry. ns, not significant; *$0.05 > p > 0.01$; **$p \leq 0.01$.

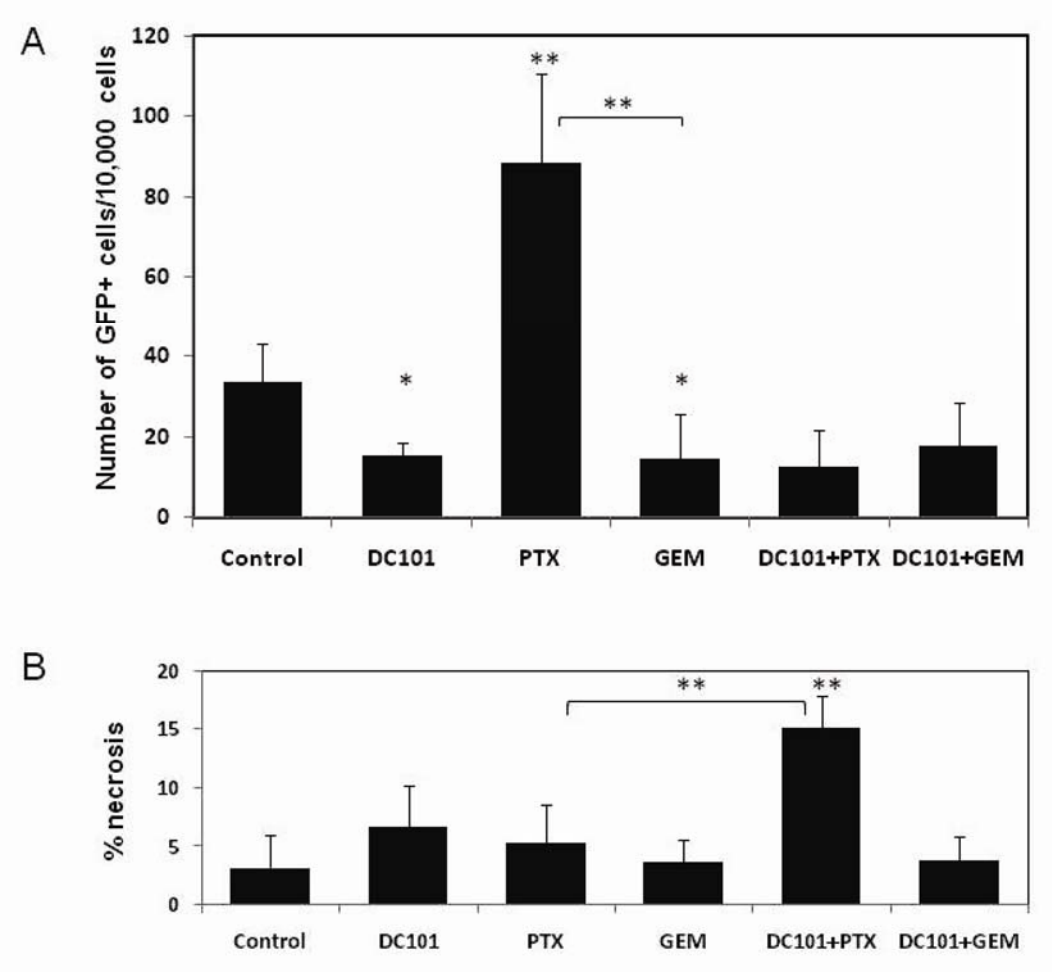

Figure S2. LLC Tumors Grown in GFP+ Bone Marrow-Transplanted C57BL/6 Mice that Subsequently Underwent Treatment with Paclitaxel or Gemcitabine in Combination with DC101

LLC tumors in GFP+ bone marrow transplanted C57BL/6 mice (n=5 mice/group)were allowed to grow until they reached 500mm^3, at which point treatments with paclitaxel, or gemcitabine in combination with DC101 were initiated.

(A) Evaluation of the number of GFP+ cells colonizing the tumor using tumors prepared as single cell suspension and evaluated by flow cytometry as described in Experimental Procedures.

(B) A summary graph for % of tumor necrosis (from Figure 4C) is presented. *$0.05 > p > 0.01$; **$p \leq 0.01$.

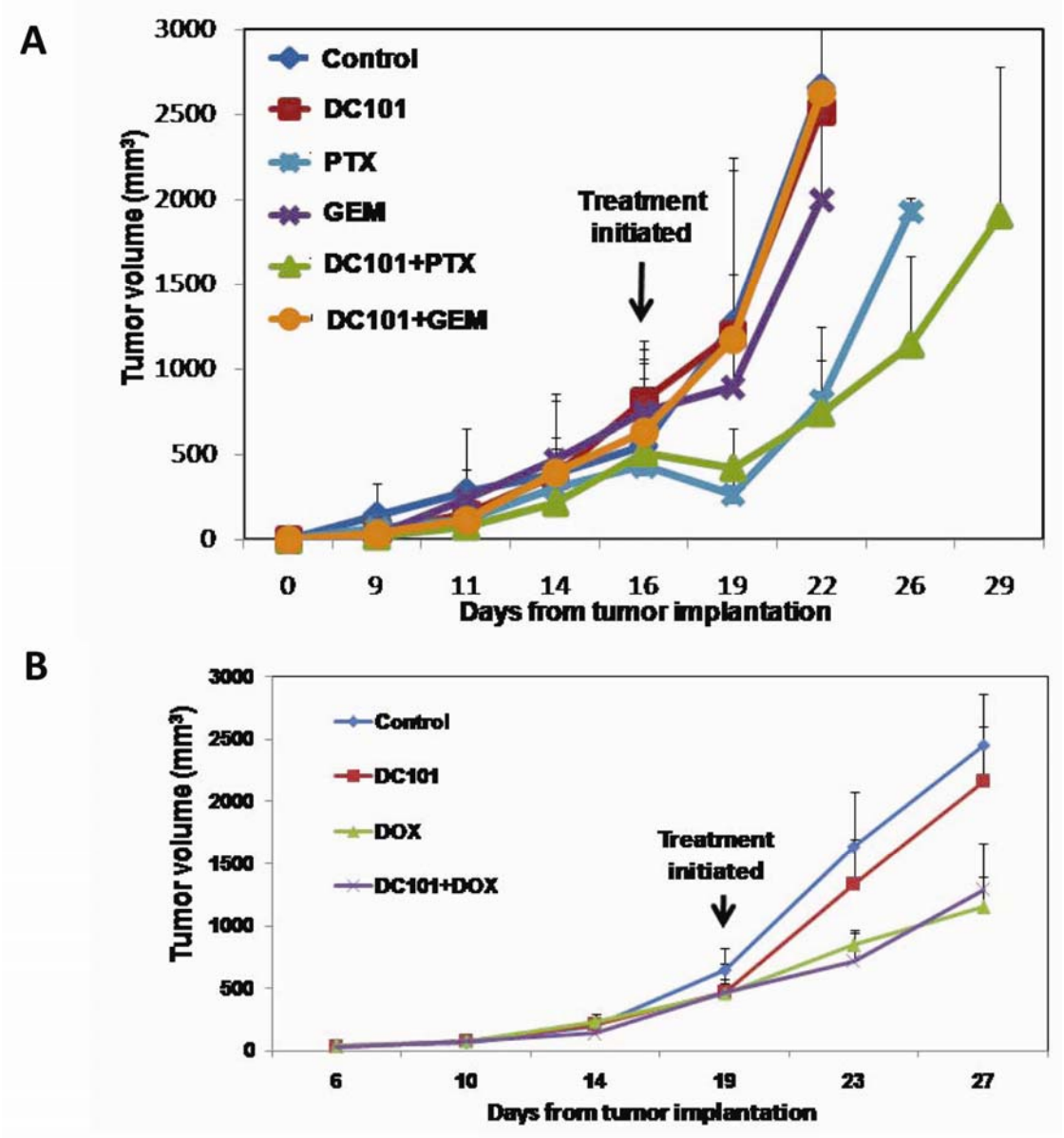

Figure S3. Long-Term LLC or B16F1 Tumor Growth in C57BL/6 Mice Treated with Paclitaxel, Gemcitabine, or Doxorubicin in Combination with DC101

(A) $0.5x10^6$ B16F1 melanoma cells were implanted in the flanks of 8-10 week old C57BL/6 mice (n=5 mice/group). Tumors were allowed to reach $500mm^3$, at which point treatment with paclitaxel, gemcitabine (administered at the MTDs) and DC101 was initiated. Tumors were measured regularly using a caliper, and tumor growth was plotted as per number of days from tumor cell implantation.

(B) $0.5x10^6$ LLC cells were implanted in the flanks of 8-10 week old C57BL/6 mice (n=4-5 mice/group). Tumors were allowed to reach $500mm^3$, at which point treatment with 12 mg/kg doxorubicin and DC101 was initiated. Tumors were measured regularly using a caliper, and tumor growth was plotted as per number of days from tumor cell implantation.

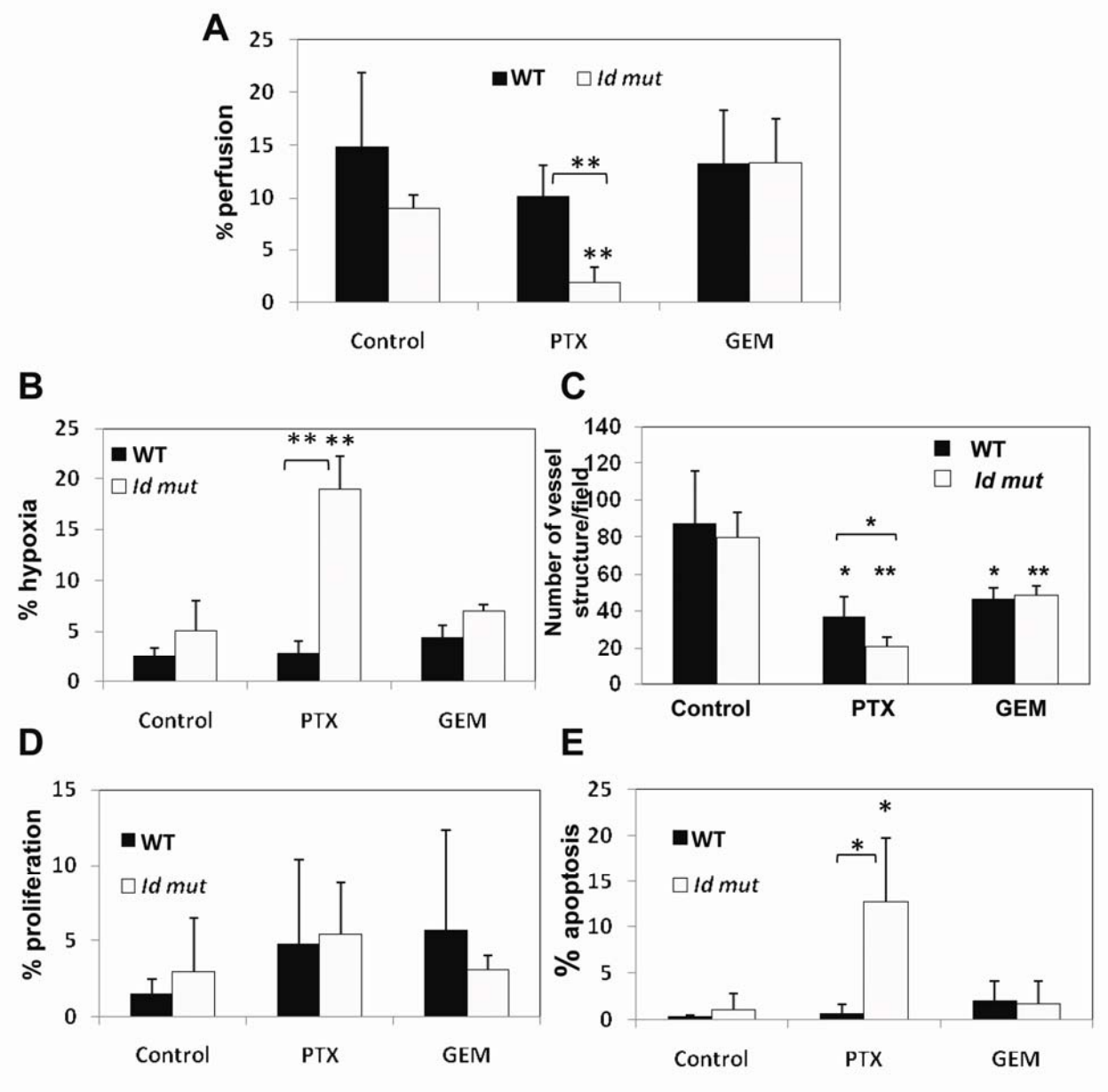

Figure S4. Evaluation of Perfusion, Hypoxia, Microvessel Density, Tumor Cell Proliferation, and Apoptosis in LLC Tumors Grown in Id Mutant and WT Control Mice after Treatment with Paclitaxel or Gemcitabine

LLC tumors from Figure 5 were evaluated for percentages of perfusion (A), hypoxia (B), microvessel density (C), tumor cell proliferation (D), and tumor cell apoptosis (E). Summary graphs are presented. *$0.05 > p > 0.01$; **$p \leq 0.01$.

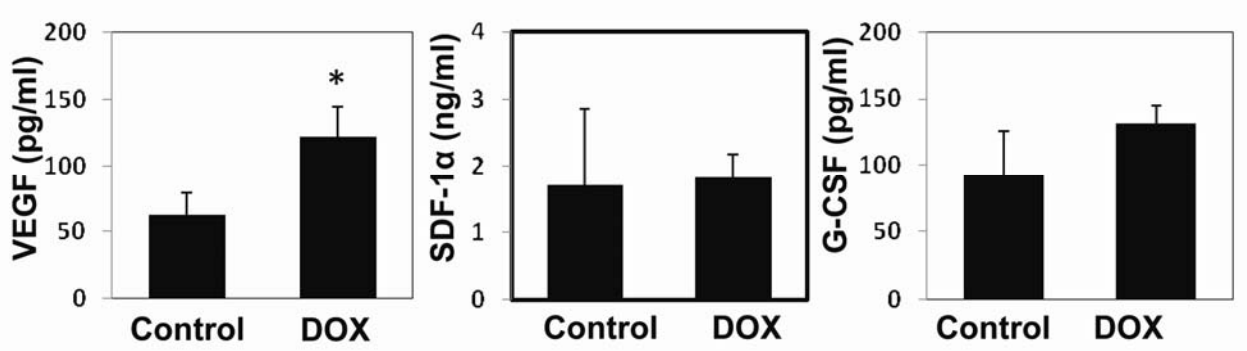

Figure S5. Evaluation of VEGF, SDF-1, and G-CSF Plasma Levels after Mice Were Treated with Doxorubicin

Non-tumor bearing C57BL/6 mice (n=4 mice/group) were treated with 12mg/kg doxorubicin (DOX). Four hours later, mice were bled by cardiac puncture and plasma was separated and analyzed using ELISAs specific for VEGF, SDF-1 and G-CSF.

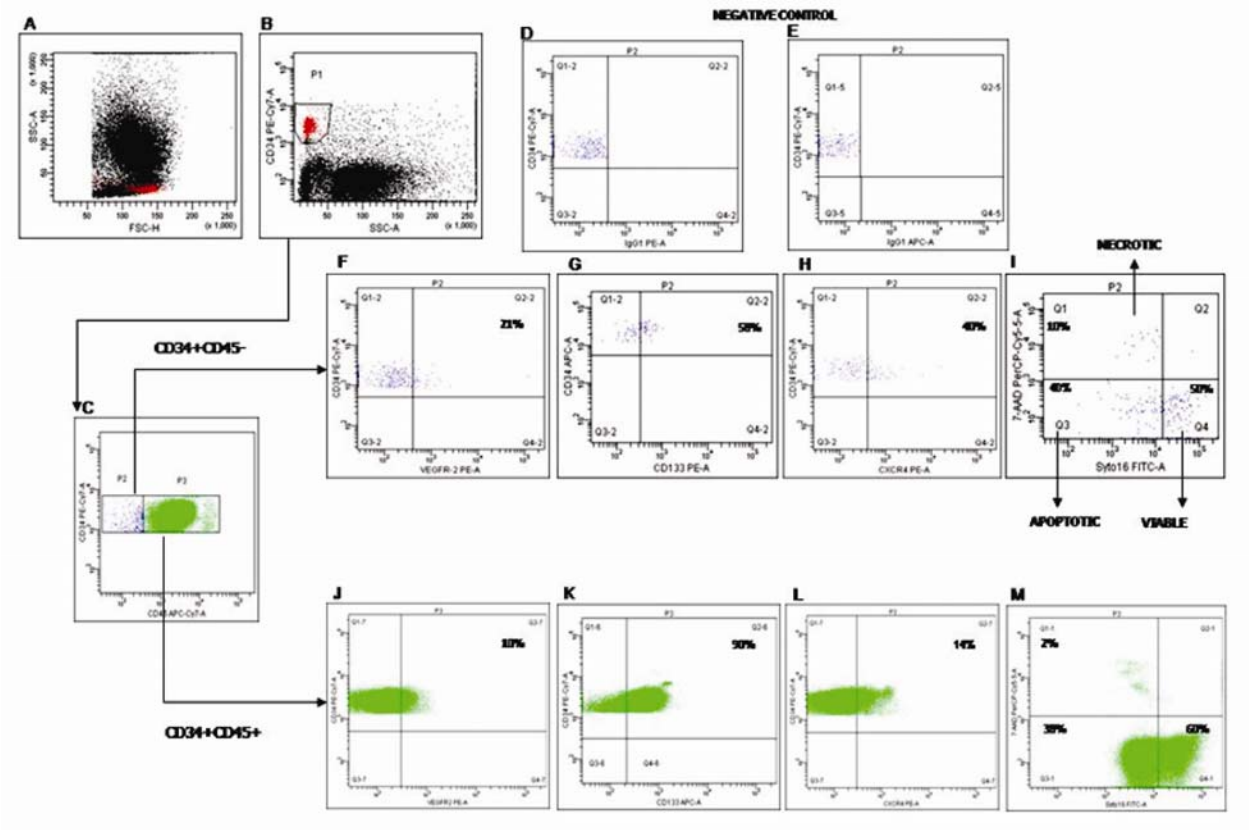

Figure S6. Representative Enumeration of CEPs Assessed by Flow Cytometry in a Breast Cancer Patient

(A) shows physical cell parameters, and the localization of CD34+ cells (in red, as gated in B) as lymphoid-like cells. (C) shows how CD34+ cells were subsequently gated and analyzed as two distinct populations of CD34+CD45- (most likely containing true CEPs according to Case et al) and CD34+CD45+ cells. (D) and (E) show the negative controls. (F), (G), and (H) show the frequency of CD34+CD45- cells expressing VEGFR2, CD133 and CXCR4, respectively. (I) shows the relative frequency of viable, apoptotic and necrotic CD34+CD45- cells as indicated by 7AAD and Syto16 staining. (J), (K), and (L) show the frequency of CD34+CD45+ cells expressing VEGFR2, CD133 and CXCR4, respectively. (M) shows the relative frequency of viable, apoptotic and necrotic CD34+CD45+ cells as indicated by 7AAD and Syto16 staining.