Tumorigenesis and Neoplastic Progression

Heterogeneity of Tie2 Expression in Tumor Microcirculation

Influence of Cancer Type, Implantation Site, and Response to Therapy

Kelly E. Fathers,* Courtney M. Stone,*
Kanwal Minhas,* Jason J.A. Marriott,*
Janice D. Greenwood,* Daniel J. Dumont,[†] and
Brenda L. Coomber*

From the Department of Biomedical Sciences, Ontario
Veterinary College, University of Guelph, Guelph; and the
Division of Molecular and Cellular Biology Research,[†]
Sunnybrook and Women's Research Institute, Toronto,
Ontario, Canada*

To evaluate the expression of the Tie2/Tek tyrosine kinase receptor in tumor blood vessels, we examined Tie2lacZ⁺/RAG1⁻ mice. There was considerable heterogeneity (Tie2-negative, Tie2-positive, or Tie2-composite blood vessels) in subcutaneous xenografts of human colorectal carcinoma (HCT116; 97.5% Tie2-positive vessels) versus human melanoma (WM115; 75.9% Tie2-positive vessels). Similar patterns of Tie2 expression occurred in abdominal metastases derived from the same cell lines. Immunostaining for endothelial markers and Tie2 revealed that endogenous protein levels corresponded with transgene activity. Endothelial cells were confirmed to be of mouse origin through triple immunofluorescence staining with mouse antiserum to human nuclei, isolectin GS-IB₄, and anti-Tie2. Similar Tie2 heterogeneity was observed in clinical specimens from a variety of human cancers, including malignant melanoma and colorectal carcinoma. We also examined the effect of Tek-Delta Fc anti-angiogenic therapy on tumor growth and Tie2 expression patterns in HCT116 and WM115 subcutaneous xenografts. Tek-Delta induced extensive tumor regression in HCT116 tumors and concomitant reductions in Tie2-expressing blood vessels. However, no significant responses were seen in Tek-Delta-treated WM115 tumors. Thus, vascular heterogeneity of Tie2 expression is cancer-type specific, suggesting that the tumor microenvironment and/or

direct cancer cell interactions influence Tie2 endothelial expression. (Am J Pathol 2005, 167:1753–1762)

Perpetual recruitment of new blood vessels (angiogenesis) is a hallmark and a prerequisite of tumor growth and progression.^{1,2} Targeting the tumor vasculature is thus an attractive anticancer treatment modality, originally expected to have almost universal efficacy across a wide tumor spectrum.² However, our previous studies demonstrated that cancer cells are heterogeneous for their dependence on the vasculature,³ which may impact responses to anti-angiogenic therapy.⁴ As well, recent work confirming vascular mimicry in a variety of tumor types⁵ and demonstrating genetic instability in tumor associated endothelium^{6,7} suggests a more complex interaction between cancer cells and tumor microcirculation during anti-angiogenic therapy than previously anticipated.

These findings may account for the mixed results of anti-angiogenic therapies seen in clinical trials to date. Despite successful preclinical studies, poor results were achieved in a Phase III trial using the vascular endothelial growth factor (VEGF) receptor-2 inhibitor SU5416 in combination with chemotherapy.⁸ Although the anti-angiogenic agent bevacizumab (Avastin) exhibits a significant antitumor effect in advanced colorectal carcinoma,⁹ it failed to increase patient long-term survival in renal cell

Supported by National Cancer Institute of Canada grants 011162 and 014104 and by the Terry Fox Foundation (to B.L.C.). K.E.F. and C.S. were recipients of Ontario Graduate Scholarships in Science and Technology.

Accepted for publication August 9, 2005

Current address of K.E.F.: Molecular Oncology Group, Royal Victoria Hospital, Montreal QC, Canada H3A 1A1.

Current address of J.D.G.: Vita-Tech Laboratories Inc., 1345 Denison Street, Markham, ON, Canada L3R 5V2.

Address reprint requests to Brenda L. Coomber, Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, N1G 2W1. E-mail: bcoomber@uoguelph.ca.

carcinoma¹⁰ or relapsed, metastatic breast cancer.² An improved understanding of both tumor biology and the mechanisms of anti-angiogenic drugs is needed to guide future clinical trials.²

The Tie2/Tek receptor and its ligands, the angiopoietins (Ang), are important for vessel remodeling, maturation, and stability.^{11–13} Angiopoietin-1 plays a role in vessel maturation through the recruitment of mural cells such as pericytes, whereas Ang2 is involved in vessel remodeling and can induce either angiogenesis or vessel regression depending on the context.^{14,15} The Tie2 receptor is ubiquitously expressed in highly vascularized embryonic and adult tissues, suggesting that Tie2 expression is a general feature of all endothelial cells.^{11,16,17} In cancer, Tie2 expression is up-regulated in various tumors, including hepatocellular carcinoma and colorectal cancer, however, it was also reported to be heterogeneously expressed in vessels of human breast and pancreatic carcinoma.^{18–21}

In this study, we examine Tie2 expression using the *Tie2lacZ*⁺/*RAG1*[−] mouse, which carries an endothelial cell-specific reporter gene construct (*lacZ*) and supports xenografts of human cancer cells. Our data provide evidence that Tie2 expression is heterogeneous in the vasculature of different types of cancer and that this heterogeneity is a function of tumor type rather than implantation site. We further show that this phenomenon is not due to vascular mimicry and is also present within human cancers. By using an anti-angiogenic agent that targets the Tie2 signaling axis, we were able to show a reduction in the proportion of Tie2-expressing endothelial cells, although the response to such treatment depended on the degree of Tie2 heterogeneity within tumors. We postulate that the source of heterogeneous responses to anti-angiogenic therapies may lie with the under-appreciated heterogeneity of tumor endothelial cells and may help explain the limited success encountered during clinical trials with some agents.

Materials and Methods

Mouse Breeding and Characterization

Both *Tie2lacZ* [FVB/N-TgN(TIE2-lacZ)182Sato] on a FVB/NJ background^{17,22} and *RAG1* null mice [B6.129S7-*Rag1tm1Mom*] on a C57BL/6J background²³ were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained under barrier conditions at the isolation facility of the Ontario Veterinary College, University of Guelph. Female homozygous *RAG1* null mice were crossed with male mice hemizygous for the *Tie2lacZ* transgene, and the progeny were bred to homozygosity at both alleles. *RAG1*[−] status was determined from blood using the Ouchterlony immunodiffusion assay and *Tie2/lacZ* status via PCR of genomic DNA according to Jackson Laboratories protocols. All mouse protocols followed Canadian Council on Animal Care guidelines and were approved by the University of Guelph Animal Care Committee.

Matrigel Implantation

Ten *Tie2lacZ*⁺/*RAG1*[−] mice were injected subcutaneously with 500 μ l of a mixture of Matrigel, basic fibroblast growth factor (4 μ g/ml), and vascular endothelial growth factor (1000 μ g/ml VEGF). After 10 days, mice were euthanized by CO₂ asphyxia; and the Matrigel plug was removed, embedded in OCT cryomatrix, and snap frozen in liquid nitrogen. Cryosections of Matrigel were stained with X-gal and either anti-von Willebrand factor (anti-vWf) or anti-CD31 as described below.

Tumor Xenografts

We established xenograft tumors in our *Tie2lacZ*⁺/*RAG1*[−] mice using the human colorectal carcinoma cell line HCT116²⁴ and the human melanoma cell line WM115.²⁵ Cells were maintained in Dulbecco's modified Eagle's medium or RPMI supplemented with 10% FBS in a humidified atmosphere of 5% CO₂. For subcutaneous xenografts, 17 mice were injected with 1×10^6 cells (WM115 or HCT116) in the right flank. Tumor growth was assessed by caliper measurements every few days, and tumor size was calculated using the equation: volume = length $\times$ width² $\times$ 0.5. Mice were sacrificed before tumor size exceeded 500 mm³, and tumor and normal tissue samples were snap frozen in OCT cryomatrix. For splenic injections, 20 mice were anesthetized with Avertin and were injected with 1×10^6 HCT116 or WM115 cells in a total volume of 50 μ l directly into the spleen. One mouse was euthanized every 2 weeks or when mice showed clinical signs of metastatic growth, and tumors were collected and snap frozen in OCT cryomatrix.

Tek-Delta Fc Trials

Subcutaneous xenografts were established as described above in 20 mice for each cancer type (WM115 or HCT116). Tumor growth was measured using calipers every few days, and when average tumor size reached 250 mm³, mice were randomly allocated into two groups: treatment and control (10 mice in each). For the treated group, mice were injected with 500 μ g of Tek-Delta Fc²⁶ i.p. every 3 days for 2 weeks (HCT116) or 3 weeks (WM115); control mice were injected with equivalent volumes of phosphate-buffered saline (PBS). For HCT116, mice were euthanized when tumor volume exceeded 1000 mm³ or after 2 weeks of therapy; for WM115, all mice were euthanized after 3 weeks of therapy. Tumors were collected as described above.

Dual Staining for X-gal and CD31 or vWF

Ten-micrometer-thick cryosections were fixed in 70% ethanol, washed in PBS, and incubated in X-gal at 37°C overnight according to established methods.²² Sections were then washed and incubated in 3% H₂O₂ in PBS for 15 minutes, followed by 10% normal goat serum for 60 minutes. After blocking, the sections were covered with rabbit anti-human vWF (1:50; Dako, Carpinteria, CA) and

incubated overnight at 4°C, followed by goat anti-rabbit hydrogen peroxidase-conjugated secondary antibody (1:300; Sigma-Aldrich, Mississauga, ON, Canada) for 30 minutes at room temperature. Sections were washed and subjected to 3-amino-9-ethylcarbazole (AEC) substrate reaction (Sigma-Aldrich), counterstained with hematoxylin, and mounted using Aquapolymount (Polyscience, Warrington, PA). Staining for X-gal and CD31 was carried out as above, with the following modifications. Blocking was with 2% bovine serum albumin (BSA) for 20 minutes, followed by 10% normal rabbit serum for 30 minutes; rat anti-mouse CD31 primary antibody (Serotec, Raleigh, NC) was used at 1:100 for overnight at 4°C, and rabbit anti-rat biotinylated secondary antibody (Vector, Burlington, ON, Canada) was diluted 1:100 for 30 minutes. Antigen detection was via the ExtrAvidin Peroxidase system (Sigma-Aldrich). Images were captured using an Olympus BX61 microscope (Olympus, Hamburg, Germany), with a Roper Scientific camera (Roper Scientific, Tucson, AZ), and MetaMorph software (Molecular Devices Corporation, Downingtown, PA).

Dual Immunofluorescence

Frozen sections were fixed at room temperature for 15 minutes in Histochoice Molecular Biology fixative (Cedarlane Laboratories, Hornsby, ON, Canada). Sections were then washed, blocked with 1% BSA for 20 minutes followed by 10% normal goat serum for 30 minutes, and incubated overnight at 4°C with a Tie2 primary antibody (Tek-4 antibody²⁷ diluted 1:200). Slides were washed and incubated with a 1:200 dilution of secondary antibody (goat anti-rat Cy3; BIO/CAN Scientific, Mississauga, ON, Canada) for 30 minutes. After washing, a 1:50 dilution of rabbit anti-vWF primary antibody was applied for 3 hours at 4°C, followed by a 1:500 dilution of Alexa fluorescent 468 goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 30 minutes at 4°C.

Formalin-fixed paraffin-embedded sections of human colorectal carcinoma, pancreatic carcinoma, prostatic carcinoma, breast carcinoma, and lung carcinoma (Peta-gen Inc., Seoul, Korea) as well as melanoma clinical specimens [courtesy of Dr. Vivi Ann Florenes (Norwegian Radium Hospital, Montebello, Oslo, Norway)] and brain glioblastoma multiforme and anaplastic astrocytoma samples (University of Toronto Nervous System Tissue Bank, Toronto, ON, Canada) were deparaffinized and immersed in sodium citrate, pH 6.0 at 95°C for 8 minutes. Sections were washed and incubated in Dako Protein block (Dako) and IT Signal Enhancer (Molecular Probes) for 30 minutes each followed by overnight incubation in a 1:150 dilution of rat anti-Tek primary antibody at 4°C and a 1:200 dilution of goat anti-rat Cy3 secondary antibody (BIO/CAN Scientific) for 30 minutes. Sections were then washed and incubated for 1 hour in a 1:200 dilution of mouse anti-CD31 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a 1:200 dilution of fluorescein isothiocyanate-conjugated rabbit anti-goat secondary antibody (Sigma-Aldrich) for 30 minutes.

Immunofluorescence for Tie2 and Isolectin GS-IB₄

Slides were fixed in 4% paraformaldehyde for 10 minutes, washed, blocked with 2% BSA and 5% normal goat serum for 30 minutes, and stained for Tie2 as described above, using a Cy3-conjugated secondary antibody. A 1:30 dilution of anti-human nuclei primary antibody (Chemicon International, Temecula, CA) was then applied, and sections were incubated overnight at 4°C, followed by 30 minutes in a 1:200 dilution of Alexa Fluor 350 goat anti-mouse secondary antibody (Molecular Probes). Sections were then washed with PBS containing calcium chloride and magnesium chloride (Sigma, St. Louis, MO), and a 1:200 dilution of Alexa Fluor 488-conjugated *Griffonia simplicifolia* isolectin GS-IB₄ (Molecular Probes) was applied for 1 hour.

Blood Vessel Assessment

To quantify Tie2 expression patterns, slides were coded and scored in a semiblinded fashion. For subcutaneous tumors, one block from each tumor was assessed. For abdominal tumors, several blocks (2–6) were collected from each tumor, resulting in 21 samples from 5 WM115 tumors and 6 samples from 3 HCT116 tumors. Sections were examined by light microscopy at ×20 magnification, and the total number of blood vessels and their Tie2 staining patterns were scored. All sections evaluated for the Tek Delta trials were scanned using Pathscan Enabler microscope slide scanner (Electron Microscopy Sciences, Hatfield, PA) and Optimas 6.2 image analysis software (MediaCybernetics, San Diego, CA), and the area morphometry was determined. The total number of blood vessels for each section was divided by the surface area to obtain the microvessel density (number of blood vessels per square millimeter). The average number of Tie2-positive, Tie2-negative, and “composite” vessels per square millimeter was also calculated for tumor sections from the Tek Delta trial.

Statistical Analysis

Normality was tested using the Anderson-Darling method. The Kruskal-Wallis test was used to examine significant differences among Tie2 status, implantation site, and tumor type ($P < 0.05$) for subcutaneous tumors. One-way analysis of variance followed by Tukey's test was used to determine significant differences in Tie2 status for the treated and untreated samples in the Tek Delta trials.

Results

Tie2 Expression in Endothelium from Transgenic Mice

To localize transgene expression in normal tissue, frozen sections were incubated with the β -galactosidase sub-

Figure 1. Histochemical and immunostaining evidence for Tie2 heterogeneity in vessels of *Tie2lacZ*⁺/*RAG1*[−] mouse tissue. Normal mouse organs (eg, liver; **A** and **B**) show robust, vessel-specific β -galactosidase activity when reacted with X-gal substrate (**A**); reaction product coincides with endogenous Tie2 protein, as revealed by immunostaining (**B**). Serial cryosections of HCT116 (colorectal carcinoma) subcutaneous tumors reacted with X-gal (**C**) and dual immunostained with anti-vWF (green) and anti-Tie2 (red) (**D**) revealed similar concordance between transgene activity and endogenous Tie2 protein. In other tumor-associated vessels, Tie2 expression (as transgene activity and endogenous protein levels) is patchy or absent (**E** and **F**). Tie2-composite vessels were also apparent in xenografts, consisting of β -galactosidase-positive endothelial cells (**arrows**) and adjacent β -galactosidase-negative endothelial cells (**arrowheads**) within the same blood vessel (**G**; red-brown reaction product indicates CD31 immunostaining). Matrigel implants were well vascularized with *lacZ*-positive angiogenic blood vessels (**arrows** in **H**). *, vessel lumen. Scale bar = 30 μ m.

strate X-gal. The highly vascularized tissues examined, such as spleen, kidney, liver, and lung, showed consistent X-gal staining in vascular structures, indicating that the transgene is well expressed and appears to be specific to the vascular endothelium (Figure 1A). Immunostaining of adjacent sections for Tie2 indicated that transgene activity reflected endogenous levels of Tie2 protein (Figure 1B). All blood vessels present within normal tissues appeared to be stained with X-gal reaction product and with an anti-Tie2 antibody, and tissue from wild-type mice showed no detectable X-gal reaction product under these conditions.

Although normal tissue showed robust staining, our initial analysis revealed that some blood vessels within the tumor were lacking transgene expression. Dual staining for either endogenous Tie2 or X-gal and endothelial-specific markers vWF or CD31 revealed that Tie2 levels and transgene expression was heterogeneous in WM115 and HCT116 xenografts (Figure 1, C–F). Tumors obtained from both subcutaneous and abdominal locations demonstrated Tie2 vascular heterogeneity; there were Tie2-positive blood vessels, Tie2-negative blood vessels, and composite blood vessels composed of Tie2-positive en-

dothelial cells as well as Tie2-negative endothelial cells (Figure 1G). Vascular heterogeneity of Tie2 from both subcutaneous and abdominal tumors suggests that Tie2 heterogeneity is not a function of implantation site. Strong β -galactosidase expression was found in all blood vessels present within the Matrigel plugs, suggesting that lack of Tie2 staining and transgene expression in some xenograft vessels is not due to their angiogenic status (Figure 1H). Triple fluorescent staining for human nuclear marker, endogenous Tie2, and isolectin GS-IB₄ revealed that these Tie2-negative cells lining the blood vessel wall were isolectin-positive mouse cells and not of human tumor origin (Figure 2).

Quantification of Tie2 Heterogeneity within Tumors

We scored xenografted tumors for the presence of Tie2-positive, Tie2-negative, and Tie2-composite blood vessels as determined by X-gal staining. Overall, Tie2-positive blood vessels made up the majority in both tumor types analyzed, but significant differences existed be-

Figure 2. A through H: Immunohistochemical staining of xenografts confirming mouse endothelial cell identity of Tie2-negative vascular cells. **Top panels:** Tie2-positive vessel, showing good correlation between GS-IB₄ isolectin staining (A) and Tie2-positive endothelial cells (B). Panel stained with anti-human nuclear antigen (C) and merged image (D) confirm the mouse origin of these vascular cells. **Dotted lines** delineate the extent of the vessel lumen in C. **Bottom panels:** Tie2-composite vessel, showing that although the vessel is completely lined by vascular endothelium (isolectin staining in E), immunostaining for Tie2 is patchy (arrows in F). Staining with anti-human nuclear antigen (G) and merged images (H) indicate that human cancer cells do not protrude or reside in the lining or wall of this vessel, thus the Tie2-negative cells are mouse endothelium. **Dotted lines** delineate the extent of the vessel lumen in (H). A and E: GS-IB₄ isolectin; B and F: Tie2; C and G: anti-human nuclear antigen; D and H: merged images. Scale bar = 20 μ m. I through K: Tie2 heterogeneity in human clinical specimens of human mammary (I), pancreatic (J), and prostate (K) carcinoma immunostained for CD31 (green) and Tie2 (red). Merged images show absence of Tie2 immunostaining in some vessels (arrowheads) and composite vessels with Tie2-positive and Tie2-negative endothelium in the same blood vessel section (arrows). I and J: Scale bar = 40 μ m; K: scale bar = 20 μ m.

tween the vessel characteristics (Table 1). Subcutaneous WM115 melanoma xenografts had significantly fewer Tie2-positive vessels than subcutaneous HCT116 xenografts (75.9% versus 97.5%); similar results were seen

with Tie2 expression patterns in abdominal tumors (Table 1). The proportion of Tie2-negative blood vessels was also significantly higher in melanoma than colorectal carcinoma xenografts (subcutaneous and abdominal), al-

Table 1. Tie2 Heterogeneity Was Assessed by Scoring Blood Vessels under Three Categories: Tie2 Positive, Tie2 Negative, and Tie2 Composite

Tumor type	Implantation site	No. of tumors	% Tie2 positive	% Tie2 negative	% Tie2 composite
WM115	Subcutaneous	9	75.9 ± 5.2 ^a	20.8 ± 4.5 ^c	3.29 ± 1.5
	Abdominal	7	71 ± 6.3 ^a	21.6 ± 5.6 ^c	6.7 ± 2.2
HCT116	Subcutaneous	5	97.5 ± 1.7 ^b	1.5 ± 0.9 ^d	1 ± 1.1
	Abdominal	3	91 ± 4.0 ^b	5 ± 1.7 ^d	4 ± 2.6

The mean values were calculated along with the standard error. The total number of blood vessels scored for WM115 and HCT116 subcutaneous tumors were 2273 and 1913, respectively, whereas the total number of blood vessels scored for WM115 and HCT116 abdominal tumors were 8783 and 2995, respectively. Values with the same superscript (a, b, c, and d) are significantly different from the same vessel class in different cancer types, but not from each other ($P < 0.05$).

though no significant differences were seen with composite vessels (Table 1). A subset of slides was scored after immunofluorescence staining for Tie2 (using Tek4 antibody) and fluorescently tagged GS-IB₄ isolectin. The average proportion of Tie2-positive, Tie2-negative, and Tie2-composite vessels in these slides was not significantly different from the average determined from the X-gal stained slides; 79.1% of vessels were Tie2 positive in immunofluorescent-stained WM115 xenografts versus 92.6% in HCT116 (compare with Table 1).

To determine whether heterogeneity of Tie2 expression is a phenomenon common to naturally occurring cancers, human clinical specimens of malignant melanoma and colorectal carcinoma were examined using dual immunofluorescence staining for Tie2 and CD31. Evidence of Tie2 heterogeneity was found in both types of cancer, and quantification of vessel characteristics revealed virtually identical patterns of Tie2-expressing vessels as seen with xenografts (Table 2). Again, there were significantly fewer Tie2-positive and significantly more Tie2-negative blood vessels in human malignant melanoma than in human colorectal carcinoma (Table 2). Interestingly, no significant differences were observed between the Tie2 staining patterns of blood vessels in human malignant melanoma when compared with the WM115 xenografts or in human colorectal carcinoma samples when compared with HCT116 xenografts. These patterns of vessel phenotype were also seen in samples of human prostate, lung, breast, and brain tumors, with the proportion of Tie2-positive vessels ranging between 80.8% and 97.4% (Table 2). These results suggest that Tie2 heterogeneity is common in certain types of human cancers, and that human cancer cells xenografted to mice can model this vascular heterogeneity.

Differential Effects of DeltaTek Fc on HCT116 and WM115 Xenografts

We next examined the influence of this vascular heterogeneity on cancer response to anti-angiogenic therapy, using the Tie2 targeting agent Tek Delta Fc in *Tie2lacZ*⁺/*RAG1*[−] mice bearing HCT116 and WM115 subcutaneous xenografts. Within the HCT116 study, the Tek-Delta treatment group grew quickly at first, but significant tumor regression began to occur by day 12 (Figure 3A). Linear regression analysis revealed significant differences between control and treatment groups at all subsequent time points ($P < 0.05$). Within the WM115 study, significant differences in tumor growth were only observed at 12 and 18 days of treatment (Figure 3B).

The blood vessels from costained sections of treated and control tumors were scored for Tie2 status as described above. For the HCT116 colorectal carcinomas, we found a significant reduction in the microvessel density (MVD) of blood vessels in treated versus control tumors (Table 3). There was also a significant decrease in the density of Tie2-positive vessels and a concomitant increase in the density of Tie2-negative blood vessels in treated compared with control tumors (Figure 4A). In contrast, for the WM115 melanomas, there were no significant differences in overall MVD between treated and control tumors, nor were there significant changes in the density of Tie2-positive versus Tie2-negative blood vessels (Table 3 and Figure 4B).

Discussion

The *Tie2lacZ* mouse developed by Dr. Thomas Sato and colleagues^{17,22} carries the bacterial *lacZ* reporter gene

Table 2. Tie2 Heterogeneity Was Assessed in a Variety of Human Clinical Specimens

Tumor type	No. of tumors	% Tie2 positive	% Tie2 negative	% Tie2 composite
Malignant melanoma	6	80.8 ± 4.04 ^a	14.4 ± 3.04	5.3 ± 1.97
Colorectal carcinoma	6	90.9 ± 2.33	2.9 ± 6.91 ^b	2.5 ± 1.24
Pancreatic carcinoma	6	82.6 ± 8.09 ^a	14.0 ± 5.76	3.33 ± 3.33
Breast carcinoma	6	92.7 ± 3.11	7.26 ± 3.11	0.00 ± 0.00
Brain*	12	96.3 ± 2.12	3.15 ± 1.90 ^b	0.55 ± 0.27
Prostatic carcinoma	6	97.4 ± 1.92	1.28 ± 1.28 ^b	0.64 ± 0.64
Lung carcinoma	6	92.0 ± 4.65	1.93 ± 1.63 ^b	6.10 ± 4.10

The mean values for average percentage of Tie2-positive, Tie2-negative, and Tie2-composite vessels were calculated along with the standard error. The total number of blood vessels counted for each tumor type ranged between 104 and 599. Values with the same superscript (a and b) are significantly different from the same vessel class in different cancer types, but not from each other ($P < 0.05$).

*Glioblastoma multiforme and anaplastic astrocytoma.

Figure 3. Effect of the Tek-Delta inhibitor on subcutaneous tumor growth, showing mean values (SE) from HCT116 (A) and WM115 (B) xenografts. Significant differences were seen by day 12 of treatment of HCT116 xenografts ($*P < 0.05$), eventually leading to tumor shrinkage in several mice. In the WM115 trial, significant differences were found between the treatment and control mice on days 12 and 18 ($*P < 0.05$), but there were no overall patterns of growth repression over the duration of this trial.

driven by a modified Tie2 promoter, thus conferring transgene expression to the endothelium. The *RAG1* null mice, on the other hand, lack mature B and T lymphocytes, because their differentiation is arrested at an early stage and subsequent V(D)J recombination does not occur.²³ By crossing these mice, we generated an immune-deficient animal carrying an endothelial reporter gene. Thus, the *Tie2lacZ/RAG1*^{-/-} mouse described here allows for the examination of tumor-related angiogenesis and Tie2 expression patterns in human xenografted tumors.

As expected, the normal tissues we examined from these mice (including spleen, kidney, lung, and liver) showed robust and consistent X-gal staining in vascular

Figure 4. Tie2 heterogeneity scores from Delta Tek Fc treatment trials. Mean values for the average density of Tie2-positive, Tie2-negative, and Tie2-composite vessels per square millimeter were plotted along with the SE for HCT116 (A), and WM115 (B) treatment trials. HCT116 control tumors had significantly higher densities of Tie2-positive blood vessels than of Tie2-negative or Tie2-composite vessels ($*P < 0.05$), and treated tumors had significantly fewer Tie2-positive blood vessels than did control tumors ($^{\S}P < 0.05$). WM115 control and treated tumors had significantly higher densities of Tie2-positive blood vessels than of Tie2-negative or Tie2-composite vessels ($*P < 0.05$), but there were no significant differences in Tie2-positive vessel density between treated and control WM115 tumors.

Table 3. MVD from Delta Tek Fc Trials

Tumor type	No. of Tumors	Groups	MVD
HCT116	10	Control	14.2 $\pm$ 4.2 ^a
		Treated	6.6 $\pm$ 1.7 ^b
WM115	10	Control	4.3 $\pm$ 0.72
		Treated	4.9 $\pm$ 0.93

The mean values were calculated along with the SEM. Significant differences were found between the control (a) and treated (b) MVD in HCT116 tumors but not between control and treated MVD from WM115 tumors ($P < 0.05$).

structures, indicating that the transgene is well expressed in the vascular endothelium. Costaining for endothelial-specific markers and X-gal revealed that only Tie2-positive blood vessels were present within the normal vasculature. These results confirm previous findings of Tie2 expression in embryonic and adult tissues,^{11,16,17} suggesting that expression of the Tie2 receptor is a general feature of all endothelial cells. Using Matrigel, we showed that angiogenic blood vessels are also Tie2 pos-

itive, in agreement with findings by Hansbury and colleagues,²⁸ who confirmed the role of Tie2 in the differentiation of endothelial cells during angiogenesis.

This being said, histochemical staining of xenografted colorectal carcinoma (HCT116) and melanoma (WM115) tumor sections revealed that not all vascular structures within the tumors were positive for the transgene. Our results imply that although the *Tie2lacZ* transgene expression is restricted to tumor vascular endothelium and shows the same staining pattern as endogenous Tie2/Tek, not all vascular endothelial cells express Tie2 to the same extent, contrary to what is seen in the vasculature of normal tissues.^{11,16,17} Tie2 heterogeneity was also reported in blood vessels of human breast cancer²⁰ and xenografted murine breast cancer,²⁹ although the extent of this heterogeneity was not quantified. Our study expands these findings and reports significant differences in vascular Tie2 expression in melanoma and colorectal carcinoma of both xenografted tumors and human clinical specimens, as well as several other types of human cancer, providing evidence that Tie2 heterogeneity occurs in a variety of cancers and is relevant to the human tumor-associated vasculature.

To evaluate whether Tie2 vascular heterogeneity is influenced by tumor implantation site (ie, whether the source of the vascular bed/tissue microenvironment influences the endothelial phenotype³⁰), HCT116 and WM115 cells were also grown as abdominal tumors after intrasplenic injection. We saw no significant differences between subcutaneous and abdominal tumors in terms of vascular Tie2 status, indicating that implantation site does not affect Tie2 expression. Significant differences in Tie2 expression patterns were still seen between the melanoma and colorectal carcinoma tumors. This reinforces our supposition that such endothelial phenotypic differences are likely due to cancer cell influences, and that the microcirculatory bed may be similar in primary tumors and their secondary metastases, at least with regard to Tie2 expression.

There is some evidence for differences in the vasculature between primary and metastatic cancers, including lower average vascular density,³¹ greater vascular volume, and increased permeability.³² However, our results reporting similar vascular phenotype in primary and metastatic tumors of the same cell line support the concept that anti-angiogenic approaches effective at primary sites may well be successful in treating metastatic disease, as has been demonstrated in other studies.^{33,34} This is significant, because metastatic lesions are historically resistant to traditional cytotoxic agents due to their phenotypically and genotypically heterogeneous population of cancer cells, their "selection" for survival through the metastatic process, and their ability to usurp homeostatic factors unique to specific organ environments.³⁵

Recent evidence suggests that some tumors may undergo "vasculogenic mimicry," wherein cancer cells line vascular channels and form a perfused network in the absence of endothelial cells.^{36,37} As indicated in aggressive human melanoma and breast carcinoma tumors, cancer cells can generate patterned networks similar to those found during vasculogenesis and can express

markers of the endothelium and their precursor cells, such as Tie1 and Tie2.^{38–40} These properties present a challenge for histochemical identification of blood vessel cells. The xenografted tumors in our study provided an opportunity to determine whether vasculogenic mimicry occurs in this setting, and we confirm that the blood vessels within our tumors are indeed lined with mouse endothelial cells by staining with fluorescently labeled mouse endothelial-specific isolectin, refuting the possibility of vasculogenic mimicry as an explanation for lack of Tie2 expression.

Tie2 expression is reported to be up-regulated in several forms of cancer, including hepatocellular carcinoma, astrocytoma, Kaposi's sarcoma, cutaneous angiosarcoma, and non-small lung carcinoma.^{18,41–43} Furthermore, Tie2 expression has been linked to metastatic potential and tumor aggression. For instance, high Tie2 expression was associated with a greater risk of metastasis in patients suffering from colorectal cancer¹⁹ and poor overall survival and high metastasis in human breast cancer.²⁰ Although the latter study found evidence of Tie2 heterogeneity, the link between this heterogeneity and overall survival or tumor aggressiveness was not examined. Using clinical specimens of human colorectal carcinoma and malignant melanoma, we have identified Tie2-negative and Tie2-composite vessels in similar proportions to that seen in our xenograft tumors. Our results highlight the need for further analysis of Tie2 status in human tumors, to better establish the relationship between Tie2 status and clinical aggressiveness of cancers.

Using an anti-angiogenic agent that targets the Tie2 signaling axis,²⁶ we examined the response rate of HCT116 and WM115 tumors, along with changes in their Tie2 expression patterns. We obtained successful regression of HCT116 tumors, similar to results previously obtained with xenografted colorectal carcinoma using antibodies and peptide-Fc fusion proteins that specifically neutralize the Ang2-Tie2 interaction.⁴⁴ In the study of Oliner et al,⁴⁴ blocking the Ang2-Tie2 interaction resulted in tumor stasis, followed by the elimination of detectable tumors in a subset of animals. Other Tie2 targeting agents, such as ExTek gave similar results to ours when targeting breast cancer, melanoma, and mammary carcinoma.^{30,45–48} ExTek blocks *in vitro*-induced angiogenesis, as does Tek-Delta,^{26,45} however, to the best of our knowledge, the influence of such approaches in the context of decreased Tie2 expression has not previously been evaluated. Ours is the first study to report a significant decrease in Tie2-positive blood vessels from Tek-Delta-treated samples, which suggests that down-regulation of Tie2 expression may play a role in the mechanism of action of Tek-Delta.

Although we saw significant tumor regression in treated HCT116 colorectal carcinoma xenografts, the xenografts of the melanoma cell line WM115 did not respond to Tie2-directed therapy. This is perhaps not surprising considering the higher degree of Tie2-negative blood vessels we observed within this tumor type (20.8% compared with 1.5% in HCT116 subcutaneous tumors). In support of this, previous studies reported that

murine mammary carcinomas lacking Tie2 expression were less responsive to adenovirally produced dominant-negative Tie2 than those expressing Tie2.²⁹ It is of interest that in our study, no significant difference was found between the density of Tie2-negative vessels in the control WM115 tumors and the treated HCT116 tumors, suggesting the possibility that Tek-Delta may inhibit Tie2 expression only to a certain basal level in these xenografts. These findings highlight the need for a more complete understanding of factors influencing tumor-associated blood vessel phenotype.

Many factors have been shown to modulate Tie2 expression, such as TNF- α ,⁴⁹ hypoxia,¹⁷ and undefined factors produced by myeloma and breast cancer cells,^{50,51} which suggest that the cancer cells can themselves regulate Tie2 expression in neighboring endothelium. Interestingly, VEGF is reported to have no effect on Tie2 expression.⁵² Tie2 expression in endothelium has also been found to be up-regulated by shear stress and mechanical stretch, suggesting that there are physical mechanisms within the tumor microenvironment that regulate and influence its expression in the vasculature.^{53,54} Our findings of distinct spatial differences in Tie2 expression, even within the same microvessel, suggest that precise local interactions between cancer cells and the endothelium (perhaps mediated by cell-cell contact) may be responsible for the heterogeneous patterns of Tie2 expression seen in solid tumors. Tie2 promoter driven constructs are frequently used to generate transgenic mice containing endothelial-specific gene sequences.^{55,56} Because our findings demonstrate that Tie2 expression is not ubiquitous in tumor endothelium, we suggest that caution is needed when interpreting results from cancer-related studies in such transgenic mice.

In summary, our results show that heterogeneous expression of the endothelial receptor tyrosine kinase Tie2 is a feature of solid tumors and that tumor responses to Tie2 directed anti-angiogenic therapy may vary based on cancer type but not tumor location. Future work is required to determine whether the degree of Tie2 heterogeneity within clinical specimens impacts tumor progression and/or responses to therapy. Studies into the molecular events occurring within the endothelial cell and the influences of the tumor microenvironment on vascular behavior are ongoing in our laboratory using this *Tie2lacZ/RAG1* null mouse, and we believe that this will prove a useful preclinical model for furthering our understanding of Tie2 biology and responses to anti-angiogenic therapy.

Acknowledgments

We thank other members of the Coomber laboratory for helpful comments and support. Special thanks to Barb Mitchell (OVC Isolation Unit) for her assistance with the mice and for performing splenic injections. Melanoma and brain tumor specimens were generously provided by Dr. ViviAnn Florenes and Dr. Abit Guha, respectively. Murine Tek Delta Fc was provided by Dr. William Fanslow (AMGEN, Thousand Oaks, CA).

References

1. Folkman J: Tumor angiogenesis: therapeutic implications: N Engl J Med 1971, 285:1182-1186
2. Kerbel R, Folkman J: Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2002, 2:727-739
3. Yu JL, Rak JW, Carmeliet P, Nagy A, Kerbel RS, Coomber BL: Heterogeneous vascular dependence of tumor cell populations. Am J Pathol 2001, 158:1325-1334
4. Yu JL, Rak JW, Coomber BL, Hicklin DJ, Kerbel RS: Effect of p53 status on tumor response to antiangiogenic therapy. Science 2002, 295:1526-1528
5. Shirikawa K, Kobayashi H, Heike Y, Kawamoto S, Brechbiel M, Kasumi F, Iwanaga T, Konishi F, Terada M, Wakasugi H: Hemodynamics in vasculogenic mimicry and angiogenesis of inflammatory breast cancer xenograft. Cancer Res 2002, 62:560-566
6. Hida K, Hida Y, Amin D, Flint A, Panigrahy D, Morton C, Klagsbrun M: Tumor-associated endothelial cells with cytogenetic abnormalities: Cancer Res 2004, 64:8249-8255
7. Streubel B, Chott A, Huber D, Exner M, Jager U, Wagner O, Schwarzingler I: Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas. N Engl J Med 2004, 351:250-259
8. Kuenen BC, Rosen L, Smit EF, Parson MR, Levi M, Ruijter R, Huisman H, Kedde MA, Noordhuis P, van der Vijgh WJ, Peters GJ, Cropp GF, Scigalla P, Hoekman K, Pinedo HM, Giaccone G: Dose-finding and pharmacokinetic study of cisplatin, gemcitabine, and SU5416 in patients with solid tumors. J Clin Oncol 2002, 20:1657-1667
9. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F: Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004, 350:2335-2342
10. Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg S: A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 2003, 349:427-434
11. Dumont DJ, Yamaguchi TP, Conlon RA, Rossant J, Breitman ML: *Tek*, a novel tyrosine kinase located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. Oncogene 1992, 7:1471-1480
12. Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qinet Y: Distinct roles of the receptor tyrosine kinases Tie1 and Tie2 in blood vessel formation. Nature 1995, 376:70-74
13. Jones N, Iljin K, Dumont DJ, Alitalo K: Tie receptors: new modulators of angiogenic and lymphangiogenic responses. Nat Rev Mol Cell Biol 2001 2:257-267
14. Lemieux C, Maliba R, Favier J, Theoret JF, Merhi Y, Sirois MG: Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflammatory responses. Blood 2005, 105:1523-1530
15. Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG: The Tie-2 ligand Angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. J Cell Sci 2005, 118:771-780
16. Schnurch H, Risau W: Expression of Tie2, a member of a novel family of receptor tyrosine kinases in the endothelial cell lineage. Development 1993, 119:957-968
17. Schlaeger TM, Bartunkova S, Lawitts JA, Teichmann G, Risau W, Deutsch U, Sato TN: Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. Proc Natl Acad Sci USA 1997, 94:3058-3063
18. Tanaka S, Sugimachi K, Yamashita Y, Shirabe K, Shimada M, Wands JR, Sugimachi K: Tie2 vascular endothelial receptor expression and function in hepatocellular carcinoma. Hepatology 2002, 35:861-867
19. Chin KF, Greenman J, Reusch P, Gardiner E, Marme D, Monson JR: Vascular endothelial growth factor and soluble Tie-2 receptor in colorectal cancer: associations with disease recurrence. Eur J Surg Oncol 2003, 29:497-505
20. Dales JP, Garcia S, Bonnier P, Duffaud F, Meunier-Carpentier S, Andrac-Meyer L, Lavaut MN, Allasia C, Charpin C: Tie2/Tek expression in breast carcinoma: correlations of immunohistochemical assays and long-term follow-up in a series of 909 patients. Int J Oncol 2003, 22:391-397

21. Dirkin AJ, Bloomston M, Yeatman TJ, Gilbert-Barness E, Cojita D, Rosemurgy AS, Zervos EE: Differential expression of the Tie-2 receptor and its ligands in human pancreatic tumors. *J Am Coll Surg* 2004, 199:724-731
22. Schlaefer TM, Ying Q, Fujiwara Y, Magram J, Sato TN: Vascular endothelial cell lineage-specific promoter in transgenic mice. *Development* 1995, 121:1089-1098
23. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE: RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 1992, 68:869-877
24. Boyd D, Florent G, Kim P, Brattain M: Determination of the levels of urokinase and its receptor in human colon carcinoma cell lines. *Cancer Res* 1988, 48:3112-3116
25. Balaban GB, Herlyn M, Clark WH Jr, Nowell PC: Karyotypic evolution in human malignant melanoma. *Cancer Genet Cytogenet* 1986, 19:113-122
26. Das A, Fanslow W, Cerretti D, Warren E, Talarico N, McGuire P: Angiopoietin/Tek interactions regulate mmp-9 expression and retinal neovascularization. *Lab Invest* 2003, 83:1637-1645
27. Yano M, Iwama A, Nishio H, Suda J, Takada G, Suda T: Expression and function of murine receptor tyrosine kinases, TIE and TEK in hematopoietic stem cells. *Blood* 1997, 89:4317-4326
28. Hansbury MJ, Nicosia RF, Zhu WH, Holmes SJ, Winkler JD: Production and characterization of a Tie2 agonist monoclonal antibody. *Angiogenesis* 2001, 4:29-36
29. Stratmann A, Ackter T, Burger A, Amann K, Risau W, Plate KH: Differential inhibition of tumor angiogenesis by Tie2 and vascular endothelial growth factor receptor-2 dominant negative receptor mutants. *Int J Cancer* 2001, 91:273-282
30. Jung YD, Ahmad SA, Akagi Y, Takahashi Y, Liu W, Reinmuth N, Shaheen RM, Fan F, Ellis LM: Role of the tumor microenvironment in mediating response to anti-angiogenic therapy. *Cancer Metastasis Rev* 2000, 19:147-157
31. Peeters CF, Westphal JR, de Waal RM, Ruiter DJ, Wobbes T, Ruers TJ: Vascular density in colorectal liver metastases increases after removal of the primary tumor in human cancer patients. *Int J Cancer* 2004, 112:554-559
32. Bhujwalla ZM, Artemov D, Natarajan K, Ackerstaff E, Solaiyappan M: Vascular differences detected by MRI for metastatic versus nonmetastatic breast and prostate cancer xenografts. *Neoplasia* 2001, 3:143-153
33. Ellis LM, Takahashi Y, Liu W, Shaheen RM: Vascular endothelial growth factor in human colon cancer: biology and therapeutic implications. *Oncologist* 2000, 5:11-15
34. Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N: Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 1995, 95:1789-1797
35. Fidler IJ: Critical determinants of metastasis. *Semin Cancer Biol* 2002, 12:89-96
36. Maniotis AJ, Folberg R, Hess A, Sefter EA, Gardner LM, Pe'er J, Trent JM, Meltzer PS, Hendrix MJ: Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol* 1999, 155:739-752
37. Papetti M, Herman IM: Mechanisms of normal and tumor-derived angiogenesis. *Am J Physiol* 2002, 282:C947-C970
38. Bissell MJ: Tumor plasticity allows vasculogenic mimicry, a novel form of angiogenic switch: a rose by any other name? *Am J Pathol* 1999, 155:675-679
39. Hendrix MJ, Sefter EA, Kirchmann DA, Sefter REB: Molecular biology of breast cancer metastasis: molecular expression of vascular markers by aggressive breast cancer cells. *Breast Cancer Res* 2000, 2:417-422
40. Shirakawa K, Kobayashi H, Heike Y, Kawamoto S, Brechbiel MW, Kasumi F, Iwanaga T, Konishi F, Terada M, Wakasugi H: Hemodynamics in vasculogenic mimicry and angiogenesis of inflammatory breast cancer xenograft. *Cancer Res* 2002, 15:560-566
41. Takahama M, Tsutsumi M, Tsujiuchi T, Nezu K, Kushibe K, Taniguchi S, Kotake Y, Konishi Y: Enhanced expression of Tie2, its ligand angiopoietin-1, vascular endothelial growth factor, and CD31 in human non-small cell lung carcinomas. *Clin Cancer Res* 1999, 5:2506-2510
42. Brown LM, Dezube BJ, Tognazzi K, Dvorak HF, Yancopoulos GD: Expression of Tie1, Tie2, and Angiopoietins 1, 2, and 4 in Kaposi's sarcoma and cutaneous angiosarcoma. *Am J Pathol* 2000, 156:2179-2183
43. Ding H, Roncari L, Wu X, Lau N, Shannon P, Nagy A, Guha A: Expression and hypoxic regulation of angiopoietins in human astrocytomas. *Neuro-oncology* 2001, 3:1-10
44. Oliner J, Min H, Leal J, Yu D, Rao S, You E, Tang X, Kim H, Meyer S, Han SJ, Hawkins N, Rosenfeld R, Davy E, Graham K, Jacobsen F, Stevenson S, Ho J, Chen Q, Hartmann T, Michaels M, Kelley M, Li L, Sitney K, Martin F, Sun JR, Zhang N, Lu J, Estrada J, Kumar R, Coxon A, Kaufman S, Pretorius J, Scully S, Cattle R, Payton M, Coats S, Nguyen L, Desilva B, Ndifor A, Hayward I, Radinsky R, Boone T, Kendall R: Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2. *Cancer Cell* 2004, 6:507-516
45. Lin P, Polverini P, Dewhirst M, Shan S, Rao PS, Peters KG: Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth. *J Clin Invest* 1997, 100:2072-2078
46. Lin P, Buxton JA, Acheson A, Radziejewski C, Maisonpierre PC, Yancopoulos GD, Channon KM, Hale LP, Dewhirst MW, George SE, Peters KG: Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. *Proc Natl Acad Sci USA* 1998, 95:8829-8834
47. Shan S, Robson ND, Cao Y, Qiao T, Li CY, Kontos CD, Garcia-Blanco M, Dewhirst MW: Responses of vascular endothelial cells to angiogenic signaling are important for tumor cell survival. *FASEB J* 2004, 18:326-328
48. Melani C, Stoppacciaro A, Foroni C, Felicetti F, Care A, Colombo MP: Angiopoietin decoy secreted at tumor site impairs tumor growth and metastases by inducing local inflammation and altering neoangiogenesis. *Cancer Immunol Immunother* 2004, 53:600-608
49. William C, Koehne P, Steffen Jurgensen JS, Grafe M, Wagner KD, Bachmann S, Frei U, Eckardt KU: Tie2 receptor expression is stimulated by hypoxia and proinflammatory cytokines in human endothelial cells. *Circ Res* 2000, 87:370-377
50. Shirakawa K, Tsuda H, Heike Y, Kato K, Asada R, Inomata M, Sasaki H, Kasumi F, Yoshimoto M, Iwanaga T, Konishi F, Terada M, Wakasugi H: Absence of endothelial cells, central necrosis, and fibrosis are associated with aggressive inflammatory breast cancer. *Cancer Res* 2001, 61:445-451
51. Giuliani N, Colla S, Lazzaretti M, Sala R, Roti G, Mancini C, Bonomini S, Lunghi P, Hojden M, Genestreti G, Svaldi M, Coser P, Fattori PP, Sammarelli G, Gazzola GC, Bataille R, Almici C, Caramatti C, Mangoni L, Rizzoli V: Proangiogenic properties of human myeloma cells: production of angiopoietin-1 and its potential relationship to myeloma-induced angiogenesis. *Blood* 2003, 102:638-645
52. Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y: Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J Biol Chem* 1999, 274:15732-15739
53. Chang H, Wang BW, Kuan P, Shyu KG: Cyclical mechanical stretch enhances angiopoietin-2 and Tie2 receptor expression in cultured human umbilical vein endothelial cells. *Clin Sci (Lond)* 2003, 104:421-428
54. Lee HJ, Koh GY: Shear stress activates Tie2 receptor tyrosine kinase in human endothelial cells. *Biochem Biophys Res Commun* 2003, 304:399-404
55. Teng PI, Dichiaro MR, Komuves LG, Abe K, Quertermous T, Topper JN: Inducible and selective transgene expression in murine vascular endothelium. *Physiol Genomics* 2002, 11:99-107
56. Peng X, Ueda H, Zhou H, Stokol T, Shen TL, Alcaraz A, Nagy T, Vassalli JD, Guan JL: Overexpression of focal adhesion kinase in vascular endothelial cells promotes angiogenesis in transgenic mice. *Cardiovasc Res* 2004, 64:42