Tumor Cell Plasticity in Ewing Sarcoma, an Alternative Circulatory System Stimulated by Hypoxia

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Abstract

A striking feature of Ewing sarcoma is the presence of blood lakes lined by tumor cells. The significance of these structures, if any, is unknown. Here, we report that the extent of blood lakes correlates with poor clinical outcomes, whereas variables of angiogenesis do not. We also show that Ewing sarcoma cells form vessel-like tubes in vitro and express genes associated with vasculogenic mimicry. In tumor models, we show that there is blood flow through the blood lakes, suggesting that these structures in Ewing sarcoma contribute to the circulation. Furthermore, we present evidence that reduced oxygen tension may be instrumental in tube formation by plastic tumor cells. The abundant presence of these vasculogenic structures, in contrast to other tumor types, makes Ewing sarcoma the ideal model system to study these phenomena. The results suggest that optimal tumor treatment may require targeting of these structures in combination with prevention of angiogenesis. (Cancer Res 2005; 65(24): 11520-8)

Introduction

Ewing sarcoma/primitive neuroectodermal tumor has been defined as a round cell sarcoma showing different degrees of neuroectodermal differentiation that present mostly in the bone or soft tissues (1). Ewing sarcoma is a relatively rare highly aggressive neoplasm with clinically evident metastatic disease at presentation in ~25% of all patients. A striking feature of Ewing sarcoma, which often occurs at adolescence (2–4), is the presence of lakes of RBC. This was first recognized by James Ewing, which led him to regard the tumor as an endothelioma (5). Furthermore, Ewing sarcoma is characterized by a translocation that involves the Flt-1 gene, usually expressed specifically by endothelial cells. Prompted by these striking features, we considered that these tumors could provide unusual insight into the relationship between a growing solid tumor and the circulation.

When a growing tumor exceeds the size of ~1 mm³, diffusion fails to provide the essential nutrients for continuous growth. Angiogenesis, a well-established paradigm in tumors, is subsequently stimulated driven by tumor-derived cytokines, such as vascular endothelial growth factors (VEGF) and fibroblast growth factors. Microvessels lined by endothelium grow into the tumor, providing a blood supply and allowing the tumor to grow beyond the limits imposed by substrate diffusion. It is observed in many tumor types that the extent of the capillary network, as assessed by the density of microvessels, is associated with poor prognosis (6, 7). There is considerable interest in the therapeutic potential of targeting the growth of new vessels (antiangiogenesis) and the patency of those that have been formed (vascular targeting).

Patients with Ewing sarcoma have previously been reported to have high levels of circulating VEGF (8). In view of this and the relatively poor prognosis of these tumors, we anticipated that the microvessel density would be high, supported by dynamic contrast enhanced magnetic resonance imaging studies (9). Surprisingly, we actually observed a relatively low microvessel density. This observation, together with the fact that the tumors contain blood lakes, led us to investigate the microcirculation of these tumors. We showed plasticity of tumor cells, using a combination of approaches, which we interpret as vasculogenic mimicry.

Vasculogenic mimicry was initially recognized in aggressive melanomas in 1999 (10), as a process in which tumor cells gain characteristics normally restricted to endothelial cells. Through this means, tumor cells could contribute to conducting blood in vascular-like structures, a process that would be independent of regular angiogenesis and endothelial cell proliferation. After these initial observations in melanoma, evidence for vasculogenic mimicry has been reported in other tumors (11–14). However, the mechanisms driving the vasculogenic mimicry process, and the contribution of the tumor cell channels to the circulation, have been uncertain.

Materials and Methods

Patient tissue materials. Tumor tissues from 33 patients from the University of Leuven and the Leiden University Medical Center were studied, who presented with Ewing sarcoma between 1987 and 2004. Most patients were included in the European Intergroup Cooperative Ewing’s Sarcoma Study and EuroEwing studies (15). Mean age was 23.8 years at diagnosis (range: 3–59 years). Patient data were available from 31 patients; 11 died before the moment of data analysis. Mean follow-up was 50 months.
and mean survival was 34 months. Twenty patients of the 23 analyzed had an 11±2 translocation. All patient materials were handled in a coded fashion according to the protocols as detailed by the Dutch association of Medical Scientific Associations.

**Immunohistochemistry.** Paraffin sections were dried for 48 hours at 37°C before staining. Histochemical staining included H&E staining and periodic acid Schiff’s (PAS) reagent staining. The amount of blood lakes and PAS-positive loops was semiquantitatively assessed by scoring 0-4 (0, absent; 1, <5%; 2, 5-20%; 3, 20-50%; 4, >50% of tissue area). For immunohistochemistry, primary antibodies used were CD31 (1:50 dilution, DAKOCytomation, Glostrup, Denmark), CD34 (1:50, Novoceastra, Valleyckenzaard, the Netherlands), Ki-67 (1:100, Labvision, Fremont, CA), VEGF (1:100 dilution, Santa Cruz, Tebu Bio, Heerhugowaard, the Netherlands), endoglin/CD105 (1:50 dilution, Monosan, Uden, the Netherlands), VE-cadherin (1:50, Cayman, Ann Arbor, MI), and tissue factor pathway inhibitor (TFPI, 1:80, American Diagnostica, Inc., Stamford, CT). After washing, the sections were incubated with antimouse immunoglobulin (1:200, DAKOCytomation) or anti-rabbit immunoglobulin biotin-labeled secondary antibody (1:200, DAKOCytomation) followed by avidin/biotin-horseradish peroxidase (DAKOCytomation) and 3,3′-diaminobenidine as substrate. For dual staining, sections were first labeled for Ki-67 using horseradish peroxidase and then CD31 and CD34 using alkaline phosphatase reaction (DAKOCytomation). Microvessel density, proliferating tumor cells, and proliferating endothelial cells were assessed by counting in quadruplicate randomly chosen fields at 200× magnification (0.25 mm²) by three independent observers. Microvessel density was assessed in Ewing sarcoma tissues and also in 117 colorectal carcinomas, 211 renal cell carcinomas, 121 breast carcinomas, and 78 melanomas.

**Cell culture and in vitro three-dimensional tube formation.** Melanoma cell lines MUM-2B, MUM-2C, C8161, and C81-61 were used. Ewing sarcoma cell lines EW-7, A673, RD-ES, and SIM/EW27 were previously characterized by Dr. O. Delattre, RD-ES was obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640, supplemented with 10% FCS and 2 mmol/L glutamine, except for C81-61 (HAM F-10) and A673 (DMEM; Life Technologies, Paisley, United Kingdom). Cells grew on standard culture dishes except for the SIM/EW27, which were grown on collagen-coated culture dishes. Human umbilical vein-derived endothelial cells were cultured on gelatin-coated culture dishes in RPMI 1640, supplemented with 20% human serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. For the three-dimensional culture, cells were plated at 100,000 cells per well on 24-well dishes coated with rat tail collagen, as described previously (10), and cultured for 10 days.

Vascularogenic tube formation was tested using a commercial Matrigel assay kit (BD Bioscet, OEMER, the Netherlands). Cells were plated at 20,000 cells per well and grown for 16 hours before calcein labeling and fluorescence microscopy. The effect of VEGF at 1, 10, and 50 ng/ml (Peprotech, Rocky Hill, NJ) or VEGF blocking antibody HuMV833 (1:50 dilution, Protein Design Labs, Inc., Fremont, CA) was assessed on MUM-2B, MUM-2C, C8161, and C81-61. Vascularogenic assay was performed using a commercial Matrigel assay kit (BD Bioscet, OEMER, the Netherlands). Cells were plated at 100,000 cells per well on 24-well dishes coated with rat tail collagen, as described previously (10), and cultured for 10 days.

**Results**

The amount of blood lakes in Ewing sarcoma correlates to clinical outcome. In a unique series of Ewing sarcoma tissue biopsy samples, selected from patients before radiation or chemotherapy was started, we observed that microvessel density in these tumors, assessed by labeling of endothelial cells (microvessel density), was relatively low compared with other tumors (Fig. 1A). This led us to hypothesize that the blood lakes we observed in 92% of these tumors might play an important role in the tumor circulation (Fig. 1B). Supporting this notion, the blood lakes were not associated with evidence of coagulation and/or local necrosis. The cells lining the blood lakes did not label with the endothelial markers, CD31 and CD34. However, they expressed CD99, a marker of Ewing sarcoma cells and light microscopic examination (Fig. 1B) and electron-microscopic examination (not shown) clearly showed that the lake-lining cells were tumor cells and not endothelial cells. The presence of tumor cell–lined blood lakes has been described as vasculogenic mimicry. Vascularogenic mimicry was originally recognized in melanoma as PAS-positive loops that contained RBC (10). Also, the presence of PAS-positive loops was examined in Ewing sarcoma, which revealed that such loops were present in 68% of the patients (Fig. 1A). This led us to hypothesize that the blood lakes we observed in 92% of these tumors might play an important role in the tumor circulation (Fig. 1B). Supporting this notion, the blood lakes were not associated with evidence of coagulation and/or local necrosis. The cells lining the blood lakes did not label with the endothelial markers, CD31 and CD34. However, they expressed CD99, a marker of Ewing sarcoma cells and light microscopic examination (Fig. 1B) and electron-microscopic examination (not shown) clearly showed that the lake-lining cells were tumor cells and not endothelial cells. The presence of tumor cell–lined blood lakes has been described as vasculogenic mimicry. Vascularogenic mimicry was originally recognized in melanoma as PAS-positive loops that contained RBC (10). Also, the presence of PAS-positive loops was examined in Ewing sarcoma, which revealed that such loops were present in 68% of the patients (Fig. 1C). Notably, all tumors with PAS-positive networks also contained blood lakes.

To address the significance of the blood lakes, we examined whether clinical outcomes correlated with the number of blood
lakes. Interestingly, the number of blood lakes, quantified as the percentage of tumor area containing lakes, was significantly higher in the patients that subsequently died (75% higher compared with the group of patients still alive at the time of data analysis, \(P < 0.05\); Fig. 1D).

**Ewing sarcoma tumors are angiogenic; lack of correlation to clinical outcome.** In tumors, we observed a high level of proliferation (mean number of Ki-67-positive tumor cells 51.4%, SD ± 30.4%). Highly proliferative tumors usually exhibit a high level of angiogenic signaling. On the other hand, the low microvessel density that we observed in the tumors suggested that these tumors might have limited angiogenic potential. We, therefore, investigated angiogenesis using several approaches and found evidence that Ewing sarcoma is highly angiogenic. First, we found that 22% of CD31/CD34–positive vessels had one or more Ki-67-positive nuclei (Fig. 2A). Second, in all Ewing sarcoma tissues, we found high mRNA expression levels of the angiogenic growth factors VEGF-A, basic fibroblast growth factor, placenta growth factor, and angiopoietin-1 comparable with those found in other angiogenic tumors, such as breast carcinoma and fibrosarcoma (Fig. 2B). Third, we observed high VEGF protein expression in ~100% of tumor cells (Fig. 2C), in line with high circulating VEGF serum levels in Ewing sarcoma patients (8). Fourth, the cell lines EW7 and RD.ES, which give rise to tumors with typical Ewing sarcoma morphology following injection into athymic mice (Fig. 2D, E). Intriguingly, in the patient tissues, neither microvessel density nor the number of proliferating endothelial cells showed significant association with clinical outcome (Fig. 2D, E).

**Ewing sarcoma cells form vasculogenic structures in vitro and express vasculogenic mimicry–related genes.** To investigate the capacity of different Ewing sarcoma cell lines to display vasculogenic mimicry in vitro, we used three-dimensional collagen matrix tube formation assays and done direct comparisons with a range of melanoma cell lines. The aggressive EW7 cell line (known to have a high tumorigenicity in mice) efficiently formed a network of tubular structures (Fig. 3A) comparable with the melanoma cell

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**Table 1. Primers used for semiquantitative and quantitative RT-PCR**

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<th>Gene</th>
<th>PCR primers (forward, reverse)</th>
<th>Amplicon size (bp)</th>
<th>Cycle no.</th>
<th>Real-time PCR primers (forward, reverse)</th>
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lines MUM-2B and C8161, which are aggressive and display vasculogenic mimicry *in vitro* (data not shown; refs. 10, 20). The less aggressive A673 and RD.ES Ewing sarcoma cell lines (known to have a low tumorigenicity in mice) showed an intermediate activity, forming a few tubular structures (Fig. 3B and C), whereas SIM/EW27 cells formed hardly any structures at all (Fig. 3D) comparable with the nonaggressive and vasculogenic mimicry–negative melanoma cell lines MUM-2C and C81-61 (not shown). Similar results were found on Matrigel (Fig. 3E–H).

Using semiquantitative RT-PCR, we found that genes associated with vasculogenic mimicry–related tumor cell plasticity in melanoma (20) were expressed in the EW7 Ewing sarcoma cell line (Fig. 3I). We went on to perform quantitative real-time RT-PCR and found that integrin α3, VE-cadherin, TFPI-1 (Fig. 3J–L), EphA2, laminin5γ2, Tie-1, neuropilin, and endoglin (data not shown) were all highly overexpressed in EW7 and melanoma cell lines MUM-2B and C8161. Expression was lower or absent in Ewing’s cell lines A673, RD.ES, and SIM-EW27 and the nonaggressive melanoma cell lines MUM-2C and C81-61, consistent with the degree of vasculogenic mimicry in three-dimensional culture (Fig. 3J–L).

To address whether these genes are expressed *in vivo* in Ewing sarcoma, we used quantitative RT-PCR to assay TFPI-1, VE-cadherin, and EphA2 in frozen material from patients and found that all three were expressed at a high level. Furthermore, using immunohistochemistry, we observed that TFPI-1/2, VE-cadherin, and EphA2 protein was present in tumor cells lining the vasculogenic mimicry structures (Fig. 2M).

**Blood lake structures in Ewing sarcoma are part of the circulation.** To study the functionality of blood lake structures *in vivo*, EW7 tumors were grown s.c. in the flank of athymic mice. Tumors grew rapidly and we observed numerous lakes, especially in the outer rim of these tumors (Fig. 4A and B), a distribution that has been previously described in melanomas (21). The cells lining these lakes were negative for von Willebrand factor and CD31. We used several approaches to investigate whether blood was flowing through these lakes. First, mice carrying EW7 tumors were i.v. injected with MECA20, an antibody specifically recognizing mouse endothelial cells. After 15 minutes, mice were sacrificed and perfused with an India ink suspension. We found ink both in MECA20 staining vessels as well as in vascular structures negative for MECA20 and directly lined by tumor cells (Fig. 4C). Second, intravital microscopy was done. The vasculogenic structures were characterized by irregular profiles and slow blood flow (Fig. 4D, a video image can be viewed on www.fdg.unimaas.nl/AngiogenesisLab). Such vasculogenic structures were not present in LS174T colon carcinoma tumors. In a third approach, the connection of vasculogenic
structures in EW7 tumors with the circulation was shown when fluorescence was visualized in the vasculogenic structures after i.v. injection of a FITC-conjugated dextran (Fig. 4E).

**Blood flow in vasculogenic structures is inefficient; role of hypoxia.** Somewhat unexpectedly, the abundant presence of blood lakes and PAS-positive loops in the patient tissues coincided with high levels of VEGF (and other angiogenic growth factors) and ongoing angiogenesis (high numbers of proliferating endothelial cells). We, therefore, considered whether VEGF is instrumental in ongoing angiogenesis (high numbers of proliferating endothelial cells). We, therefore, suggest that VEGF might be involved in driving vasculogenic mimicry. To test this idea, the expression of the above-mentioned genes that are involved in vasculogenic mimicry was investigated in aggressive and nonaggressive tumor cell lines cultured under hypoxic and standard conditions. We found that culturing for 16 hours under hypoxia significantly increased the expression of laminin5γ2, EphA2, Tie-1, and TFPI-1 (for all these molecules p < 0.01) in all cell lines tested (Fig. 5L).

**Discussion**

Despite the clinically aggressive behavior of Ewing sarcoma, a relatively low number of microvessels was observed in this tumor type compared with other malignancies. We showed using several techniques that this was not due to a low angiogenic potential. Instead, the high angiogenic potential was illustrated by the high numbers of proliferating endothelial cells (although total number of vessels was low) and the high expression level (both RNA and protein) of angiogenic growth factors in the patient tissues as well as in the cell lines. The low amount of regular blood vessels, together with the abundant presence of blood lakes and PAS-positive loops (vascular-like tube formation by tumor cells), suggested a contribution of the blood lakes to circulation that might be considered vasculogenic structures as described earlier (10, 12, 13). We, therefore, suggest that Ewing sarcoma tumor cells cooperate in the formation of a circulatory system such as has been described as vasculogenic mimicry in aggressive melanoma. Interestingly, whereas in most tumors vasculogenic mimicry...
Figure 3. Ewing sarcoma cells form tubular structures in vitro and express vasculogenic mimicry–specific genes. Ewing sarcoma cell lines EW7 (A), A673 (B), RD.ES (C), and SIM-EW27 (D) in three-dimensional culture on collagen type I matrix. EW7 cells (E), A673 Ewing sarcoma cells (F), MUM-2B aggressive melanoma cells (G), and MUM-2C nonaggressive melanoma cells (H) on a Matrigel three-dimensional matrix stained with calcein. I, gene expression measured by semiquantitative RT-PCR in the Ewing sarcoma cell lines compared with the vasculogenic mimicry–positive melanoma cell line MUM-2B and the vasculogenic mimicry–negative cell line MUM-2C. The housekeeping gene GAPDH was used to control for equal loading. Quantitative real-time RT-PCR confirmation of VE-cadherin (J), integrin α3 (K), and TFPI-1 (L) gene expression of VE-cadherin in melanoma and Ewing sarcoma cell lines. The Ct values are corrected for the expression of housekeeping genes. Statistical significance determined by Mann-Whitney test. M, staining of Ewing sarcoma tissue sections with TFPI, VE-cadherin, and EphA2 antibodies. Bar, 100 μm [A, E (for A-H), and M].
Vasculogenic mimicry is present in 10% to 40% of cases, Ewing sarcoma is the first tumor in which vasculogenic mimicry is so abundantly present (blood lakes in 92% of cases). In Ewing sarcoma, the amount of blood lakes and presence of PAS-positive loops, in contrast to the classic angiogenesis variables, correlated with survival, confirming that vasculogenic mimicry is an indicator of poor prognosis (10, 25).

The vascular-like tube formation by Ewing sarcoma tumor cells was confirmed in a three-dimensional culture system using human cell lines, such as EW7, grown on a collagen matrix. Aggressive EW7 Ewing sarcoma cells rapidly formed vascular-like tubes in this system. In addition, EW7 cells injected into athymic mice gave rise to tumors with Ewing sarcoma morphology and blood lakes in vivo.

Furthermore, based on the similarities with the gene expression profiles in vasculogenic melanoma cells (13, 26), it was suggested that tube formation in Ewing sarcoma was due to a similar plasticity and dedifferentiation of tumor cells, which gained an endothelial phenotype. We showed that the genes involved in this process, such as TPF1s, EphA-2, and VE-cadherin (10, 27), are also highly expressed in the patient tissues and aggressive vascular-like tube-forming EW7 cells. In contrast, less aggressive Ewing sarcoma cells did not or less efficiently form these tubes and do not overexpress these genes. It is interesting in this context that one of the diagnostic indicators for Ewing sarcoma is a translocation of chromosomes 11 and 22, which involves the Fli-1 gene on chromosome 11 and the EWS gene on chromosome 22 (28, 29). In diagnostic pathology, Fli-1 antibodies are used as markers for Ewing sarcoma and for endothelial cells or vascular tumors. The protein that is formed by the Fli-1 gene is usually specifically expressed by endothelial cells. This translocation might play a role in the tumor cell plasticity seen in Ewing sarcoma.

Critical proof for the contribution of nonendothelial cell–lined vascular structures in circulation was the demonstration of blood flow in these structures. Using intravital microscopy and immunohistochemistry after injection of antiendothelium antibody and India ink, we were able to show blood flow in the nonendothelial cell–lined vascular structures. This blood flow was observed to be very slow, which urged us to study oxygenation in the tumor tissue. When assessing the Ewing sarcoma tissue samples for hypoxia, we found that the tumor cells surrounding the blood lakes did express HIF1α, a transcription factor known to play a role in the expression of VEGF, as well as GLUT1, indicative of inefficient oxygen delivery by these structures. Possible explanations for this would include the very slow blood flow through these channels, and/or the possibility that they act primarily as a circulatory system draining blood from the tumor.

It is likely that the hypoxia that is induced in these tumors has led to the high expression of VEGF (and maybe other angiogenic factors) and resultant endothelial cell proliferation as observed in the patient tissues. An important possibility is that vascular-like tube formation by tumor cells might be induced by VEGF or other angiogenic factors. In fact, tube formation has previously been reported to be enhanced by VEGF in vitro (30). In contrast, in our study, we were not able to show that VEGF enhances or induces vascular-like tube formation by tumor cells. Although this could reflect the difference in tumor models and assay systems, we were also not able to show differences in expression of the genes involved in tube formation in response to VEGF (Fig. 5).

Therefore, we favor the view that in Ewing sarcoma, blood lake and PAS-loop formation is not induced or supported by VEGF. However, we did find an increased expression of genes involved in vasculogenic mimicry when cells were cultured under low oxygen tension. Thus, we anticipate that hypoxia via induction of HIF1α is able to enhance vasculogenic mimicry. Interestingly, both HIF1α and vasculogenic mimicry associated genes (i.e., EphA2 and laminin-5/2) signal via the PI3K pathway (31, 32).

Furthermore, blockade of this signal transduction pathway blocks vasculogenic mimicry and the expression of the genes involved in this process (33). In addition, Tie-1 can be up-regulated by HIF1α (34).

The results of this study strongly suggest that plasticity of Ewing sarcoma tumors is associated with the contribution of tumor cells to contribute to circulation. This presumably explains, at least in part, why the microvessel density is unusually low for such an aggressive tumor. This scenario may have effect on the treatment of tumors with angiogenesis inhibitors that act directly on endothelial cells. As we have shown before, dedifferentiating tumor cells do not acquire sensitivity to angiogenesis inhibitors (35), suggesting that an antiangiogenesis protocol may lead to only a partial regression of the tumor. Because vascular-like tube formation is much less frequent and/ or less well recognized in other tumor types, it is possible that there is an important relationship between these structure and response to therapy, which has been overlooked in trials with angiogenesis inhibitors. Up to now, no data are available on such trials in Ewing sarcoma patients. We suggest that it would be particularly informative to seek a relationship between the presence of vasculogenic structures and the response to antiangiogenesis therapy. Furthermore, an interesting possibility is that antiangiogenesis therapy may result in a selective growth advantage for cells exhibiting vasculogenic mimicry, allowing drug-induced resistance to occur.

It seems likely that angiogenesis therapy may be more effective when combined with other forms of cancer therapies to eradicate vasculogenic tube formation, explaining the good results of combination between antiangiogenesis therapy and conventional angiogenesis inhibitors (35), suggesting that an antiangiogenesis protocol may lead to only a partial regression of the tumor. Because vascular-like tube formation is much less frequent and/ or less well recognized in other tumor types, it is possible that there is an important relationship between these structure and response to therapy, which has been overlooked in trials with angiogenesis inhibitors. Up to now, no data are available on such trials in Ewing sarcoma patients. We suggest that it would be particularly informative to seek a relationship between the presence of vasculogenic structures and the response to antiangiogenesis therapy. Furthermore, an interesting possibility is that antiangiogenesis therapy may result in a selective growth advantage for cells exhibiting vasculogenic mimicry, allowing drug-induced resistance to occur.

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cancer therapies in (pre)clinical studies (36–38). The Ewing sarcoma model with its abundant vasculogenic structures may be an ideal model to develop and test therapies designed to counteract vasculogenic tube formation by attacking tumor cells that take part in the formation of vascular lakes (e.g., through CD99-directed therapy; ref. 39).

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Figure 5. Blood lakes and hypoxia. A, Ewing sarcoma H&E-stained tumor section. B to D, CD31, HIF1α, and GLUT1 stainings, respectively. Arrows, regular blood vessels. E, pimonidazole adduct formation around blood lakes in a Ewing sarcoma mouse tumor. F, VEGF regulation of tube formation by EW7 cells cultured on Matrigel. G, regulation of vasculogenic mimicry–associated gene (TFPI-1) expression in EW7 by incubation for 48 hours in 10 ng/ml VEGF, MUM-2B, and MUM-2C cells. H, incubation of EW7 cells on a three-dimensional matrix with blocking VEGF antibody as a positive control human umbilical vein–derived endothelial cells (HUVEC) were used. Regulation of expression of vasculogenic mimicry–associated genes (laminin 5;2 (I), Tie-1 (J), TFPI-1 (K), and EphA2 (L)) by hypoxia in EW7, A673, and C8161 cells. *, P < 0.05. Bar, 50 μm.

References


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