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ORIGINAL ARTICLE

EMP2 regulates angiogenesis in endometrial cancer cells through induction of VEGF

LK Gordon[1], M Kiyohara[2], M Fu[2], J Braun[2,3], P Dhawan[4], A Chan[1,2], L Goodglick[2,3] and M Wadehra[2,3]

Understanding tumor-induced angiogenesis is a challenging problem with important consequences for the diagnosis and treatment of cancer. In this study, we define a novel function for epithelial membrane protein-2 (EMP2) in the control of angiogenesis. EMP2 functions as an oncogene in endometrial cancer, and its expression has been linked to decreased survival. Using endometrial cancer xenografts, modulation of EMP2 expression resulted in profound changes to the tumor microvasculature. Under hypoxic conditions, upregulation of EMP2 promoted vascular endothelial growth factors (VEGF) expression through a HIF-1α-dependent pathway and resulted in successful capillary-like tube formation. In contrast, reduction of EMP2 correlated with reduced HIF-1α and VEGF expression with the net consequence of poorly vascularized tumors in vivo. We have previously shown that targeting of EMP2 using diabodies in endometrial cancer resulted in a reduction of tumor load, and since then we have constructed a fully human EMP2 IgG1. Treatment of endometrial cancer cells with EMP2-IgG1 reduced tumor load with a significant improvement in survival. These results support the role of EMP2 in the control of the tumor microenvironment and confirm the cytotoxic effects observed by EMP2 treatment in vivo.

INTRODUCTION

Sprouting angiogenesis—the process by which new blood vessels grow from existing ones—is a ubiquitous phenomenon in health and disease. It has a pivotal role in diverse processes from embryo development to wound healing to tumor growth.[1] Regarding tumor angiogenesis, it has been shown that solid tumor growth depends on successful neovascularization and several factors, the most notable being vascular endothelial growth factors (VEGFs), have been shown to promote tumor angiogenesis. VEGFs were first described as a potent vascular permeability factor (VPF) secreted by tumor cells that stimulate a rapid and reversible increase in microvascular permeability without mast cell degranulation or endothelial cell damage.[2] Its importance in tumor biology has led to the development of several drugs, the most notable being the recently FDA-approved bevacizumab (Avastin; Genentech, San Francisco, CA, USA)—a humanized anti-VEGF antibody, as anticancer agents.[3,4]

VEGF polypeptides belong to the PDGF family of growth factors, and are perhaps the most important players that regulate vessel formation.[3] VEGFs are encoded by a family of genes that includes VEGF-A, -B, -C, -D and placental growth factor.[5] They are dimeric cysteine-linked secreted glycoproteins with a molecular weight of ∼40 kDa. Produced in response to hypoxia, specific growth and differentiation factors, and by oncogenes, VEGFs are produced by many cell types, including tumor cells.[5,6] In tumors, VEGF-A appears to be most potent angiogenic of the vascular growth factors,[7] and its secretion has been shown to be critical for tumor growth.[3,7] Thus, understanding the mechanisms that control angiogenesis, and in particular that control VEGF-A expression, are of paramount importance in tumor biology.

Epithelial membrane protein-2 (EMP2) is a tetraspan protein of the GAS-3/PMP22 family. Concordant with the role of tetraspans, EMP2 is thought to curate molecules on the plasma membrane to regulate the activity of specific signaling complexes. EMP2 coordinates the activity of select integrin isoforms, coalescing in the activation of FAK and Src.[8–10] Recently, EMP2 has also been revealed as a novel oncogene upregulated in a number of tumors including endometrial,[11] ovarian[12] and gliomas.[13] Upregulation of EMP2 expression has been associated with tumor progression, invasion and poor patient prognosis.[11,14]

To date, the mechanism of how changes in EMP2 levels affect the tumor microenvironment has not been evaluated. Using a model for endometrial cancer, we determined that an important function of EMP2 is as a regulator of angiogenesis. We demonstrate that increased EMP2 promotes endothelial cell tube formation through increased VEGF-A expression. Therapeutic targeting of EMP2 using monoclonal antibodies reduces tumor vasculature, identifying a novel mechanism of reducing tumor load through indirect control of VEGF-A.

RESULTS

EMP2 IgG1 improves endometrial cancer survival

We have previously shown that anti-EMP2 diabodies reduce tumor load in HEC1A xenografts.[15] Since this original study, a recombinant EMP2 IgG1 antibody has been designed and shown to be therapeutically beneficial in mouse models of breast cancer (Fu and Wadehra, submitted). To determine the efficacy of EMP2 IgG1 for endometrial cancer, subcutaneous xenografts using the HEC1A/EMP2 cell line were created. Systemic

[1]Department of Ophthalmology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA; [2]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA; [3]Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA and [4]Department of Surgery, Harbor UCLA Medical Center, Torrance, CA, USA. Correspondence: Dr M Wadehra, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA.
Email: mwadehra@mednet.ucla.edu

weekly injections of EMP2 IgG1 reduced tumor load compared to control IgG (Figure 1a). This reduction in tumor load translated into a significant increase in survival for mice treated with EMP2 IgG1 (Figure 1b; $P = 0.02$). Even after 84 days, surviving mice showed no measurable change in tumor size. Upon histologic examination, central necrosis was prominent in the tumors after anti-EMP2 antibody treatment compared with the control (Figure 1c). Although this can be attributed partially to higher interstitial pressure and poorer blood flow at the center of tumors,[16,17] significantly less necrosis was visible in tumors with control IgG antibodies treatment. To analyze these alterations in tumor vasculature, immunostaining for vasculature was performed using *Lycopersicon esculentum* lectin on surviving clusters of tumor cells. Lectin immunostaining studies confirmed the marked loss of vessels during anti-EMP2 treatment in areas of viable tumor (1D).

To further examine whether the reductions in tumor vascularity from EMP2 treatment were due to vessel regression or reduced angiogenesis, tumors were stained with Masson's trichrome to detect the presence of collagen sleeves[18,19] in necrotic areas (Figure 1e). All treatment groups had scattered fragments of basement membrane (Figure 1e, arrowheads), but empty basement membrane sleeves were more abundant in areas of necrosis after the anti-EMP2 antibody than control treatments. This suggests that blockade of EMP2 is a novel mechanism to reduce tumor neovascularization.

Tumor-associated vasculature

Recent studies have shown that EMP2 expression is upregulated in endometrial tumors and that its expression alters tumor cell development.[11,14] Studies have also shown that modulation of EMP2 does not significantly alter tumor cell proliferation.[9] Therefore, we postulated that it may be an important regulator of the tumor cell microenvironment. To test this idea, tumors were created from endometrial cancer cells that overexpressed EMP2 (HEC1A/EMP2), expressed a vector control (HEC1A/V) or expressed a ribozyme to reduce its levels (HEC1A/RIBO). Masson's trichrome staining of xenografts suggested that EMP2 expression altered tumor vasculature (Figure 2a). HEC1A/EMP2 tumors were highly vascularized, whereas tumors with reduced EMP2 (HEC1A/RIBO) levels formed small tumors with poor vasculature and large areas of necrosis.

To confirm that EMP2 levels correlated with increased numbers of blood vessels, tumors were stained with *L. esculentum* lectin, which binds uniformly to the luminal surface of the endothelium,[20] and DAPI. HEC1A/EMP2 tumors showed increased tumor-associated vasculature compared with the HEC1A/V tumors. Similar staining of HEC1A/RIBO tumors showed poor tumor vasculature with some background staining in the areas of necrosis (Figure 2b). Xenografts were also stained with CD34 antibodies (Figure 2c). Concordantly, quantitation of staining revealed a significant correlation between EMP2 expression and CD34$^+$ cells (Figure 2d).

EMP2 expression promotes endothelial cell tube formation

We used several approaches to investigate whether and how EMP2 could regulate the behavior of endothelial cells. Initially, the chemotactic response of human umbilical vein endothelial cells (HUVECs) to supernatants from EMP2-modified cell lines was tested using Boyden chambers. Conditioned media was collected from cells grown under hypoxic conditions for up to 24 h. Conditioned media from cells that overexpressed EMP2 significantly enhanced directional migration compared to control cells ($P = 0.04$; Figure 3a). Reduction in EMP2 expression further reduced cell migration by two-fold over control cells ($P = 0.03$). To confirm that EMP2 expression altered endothelial cell migration, a 'scratch' test was performed on a confluent
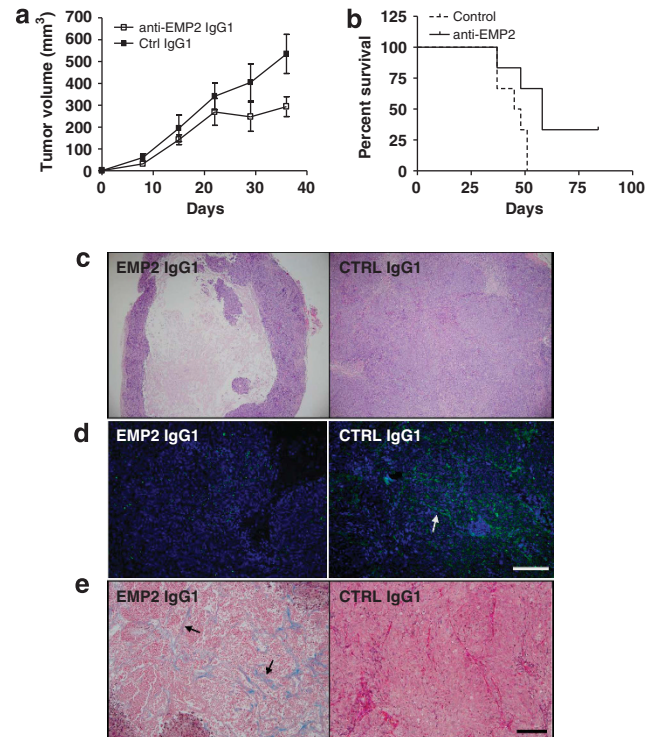

Figure 1. EMP2 IgG1 treatment reduces tumor load. (**a**) HEC1A/EMP2 cells were injected subcutaneously into nude BALB/c female mice. When tumors reached 4 mm^2, control of EMP2 IgG1 antibodies were systemically injected at 10 mg/kg. Mice were treated weekly, and tumor volume was monitored using calipers. $N = 6$. *$P < 0.05$. (**b**) Kaplan–Meier survival analysis of the outcome of athymic mice treated with anti-EMP2 IgG1 or a control antibody. $N = 6$; $P = 0.02$. (**c**) Treated tumors were harvested and fixed. Tumors were visualized using hematoxylin and eosin. Magnification: $\times 4$. (**d**) Neovascularization of HEC1A/EMP2 tumors treated with EMP2 IgG1 or control antibodies was visualized using *L. esculentum* lectin and DAPI in surviving tumor clusters. (**e**) Masson's trichrome stain was used to identify basement membrane sleeves in central areas of the tumor. $N = 3$, with a representative image shown. Magnification: $\times 10$.

monolayer of HUVEC cells. Concordant with the previous results, an EMP2 dose-dependent response was also observed using conditional medium from hypoxic HEC1A/EMP2, HEC1A/V or HEC1A/RIBO cells (Figure 3b). No statistically significant differences were observed from conditioned media collected from normoxic cells (data not shown).

To determine whether EMP2 altered the functional behavior of endothelial cells, HUVEC cells were placed on a basement membrane matrix to induce capillary-like tube formation.[21] Cells were incubated in hypoxic cultured supernatants from HEC1A/EMP2, HEC1A/V and HEC1A/RIBO (Figure 3c). An EMP2-dependent response was observed in capillary-like tube formation, as HEC1A/EMP2 induced more tube formation and tubes with a greater diameter than HEC1A/V (Figures 3d and e). Reduction in EMP2 expression in HEC1A cells further reduced the number of tubes formed compared with HEC1A/V, suggesting that EMP2 expression is necessary for endometrial tumor angiogenesis. This effect was also observed when EMP2 levels were reduced using shRNA, and although a similar trend in capillary-like tube formation was observed from normoxic cultured supernatants between HEC1A/EMP2 and HEC1A/V, these results were not statistically significant (data not shown). No significant difference in HUVEC cell proliferation was observed within the experimental window (data not shown).
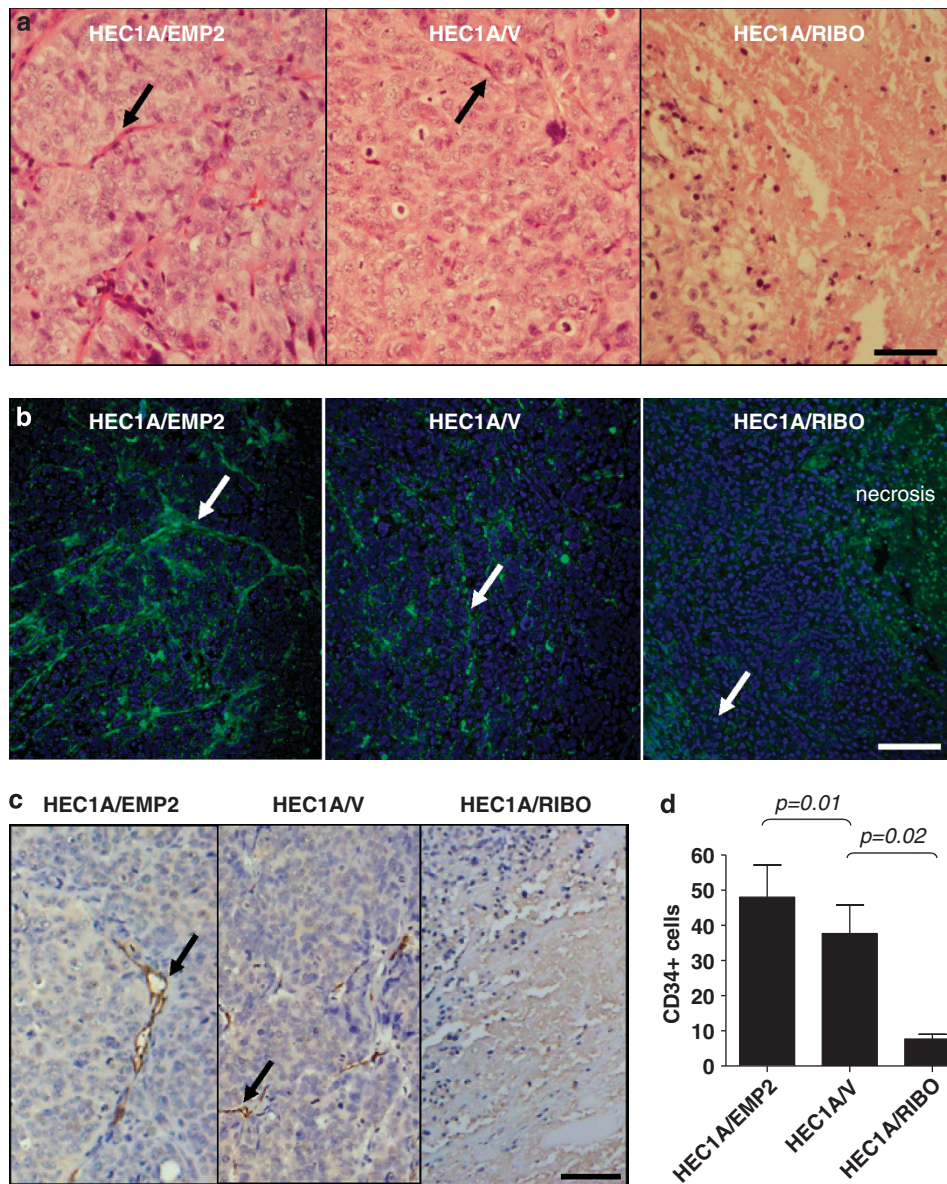
Figure 2. EMP2 expression increases tumor vasculature. (**a**) 1×10^6 HEC1A/EMP2, HEC1A/V or HEC1A/RIBO cells were injected subcutaneously into Balb/c nude mice. After 30 days, tumors were harvested, fixed and stained with Masson's trichrome. $N = 6$. (**b**) Tumors were stained using *L. esculentum* lectin and DAPI. (**c**) Tumors were stained for CD34 expression. In all images, arrows highlight tumor vasculature. Magnification: $\times 20$. Scale bar $= 100 \mu m$. (**d**) The numbers of CD34$^+$ blood vessels in six high-power fields ($\times 200$) from at least two independent tumors were counted and averaged. Bars, mean $\pm$ s.e.

The reduction in vessel formation in HEC1A/RIBO and HEC1A/sh911 suggested that EMP2 in part regulated the tumor microenvironment via new blood vessel formation. In order to link anti-EMP2 treatment with changes in neovascularization, we initially treated HEC1A/EMP2 cells for 12–15 h under hypoxic conditions with anti-EMP2 IgG1 or control antibodies to determine their effect on capillary-like HUVEC tube formation, as no cellular toxicity was visible at this early time point. EMP2 IgG1 treatment was sufficient to reduce HUVEC tube formation *in vitro* in a dose-dependent manner (Figure 3f).

To determine if the effects of EMP2 on endothelial cells are cell type specific, similar experiments were performed on primary human aortic endothelial cells (HAEC). Using supernatant from hypoxic HEC1A/EMP2, HEC1A/V or HEC1A/RIBO as a chemoattractant, Boyden chamber assays were performed on HAEC. Similar to results using HUVEC cultures, tumor expression of EMP2 promoted HAEC invasion (Figure 4a). These combined results suggest that EMP2 upregulation leads to an increase in pro-angiogenic events. Several cellular and molecular changes have been shown to promote tumor angiogenesis,[22] with the most potent inducer being VEGF.[23] In order to determine if VEGF contributed to HAEC invasion, tumor cell supernatants were incubated with bevacizumab, a monoclonal antibody to VEGF. Treatment with bevacizumab reduced HAEC migration to control levels, suggesting that EMP2 may regulate VEGF expression (Figure 4a).

EMP2 regulates VEGF expression

In order to determine if EMP2 expression altered VEGF expression and secretion, cells were grown in normoxia or placed in a hypoxic chamber for 24 h. VEGF expression was below detection under normoxic conditions. However, when cells were placed in hypoxia,
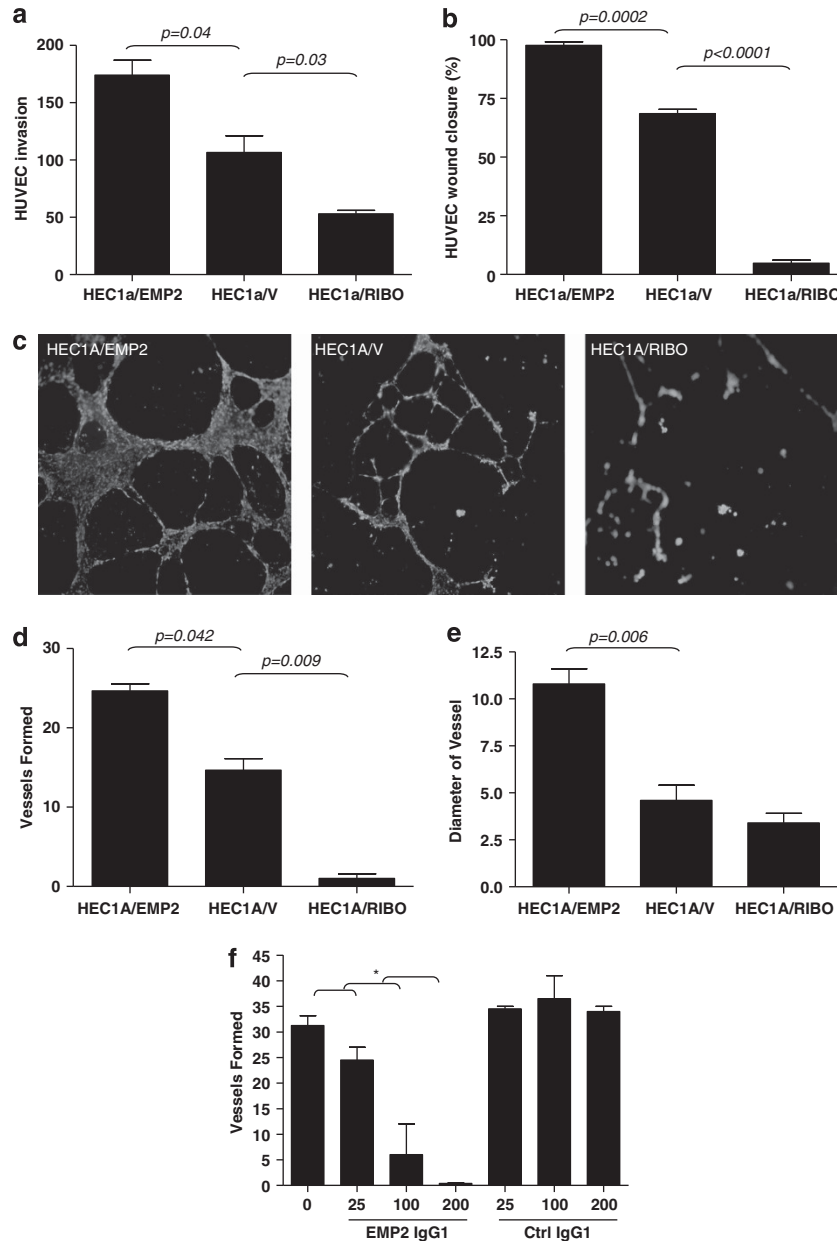
Figure 3. EMP2 promotes angiogenesis. (**a**) Chemotactic effects on the migration of HUVECs were measured using a standard Boyden chamber assay. HUVEC cells were stimulated to migrate in response to cultured media from hypoxic HEC1A/EMP2, HEC1A/V or HEC1A/RIBO cells. Experiments were repeated three times with data representing the mean ± s.e. (**b**) HUVEC cells' migration was measured using a 'scratch' wound healing assay. Endothelial cells were cultured in hypoxic tumor cell supernatant, and wound closure was measured using microscopy. (**c**) HUVEC cells were plated on low growth factor matrigel in the presence of cultured media from hypoxic HEC1A/EMP2, HEC1A/V or HEC1A/RIBO cells. The experiment was repeated at least three times, and a representative image is shown. Capillary-like tube formation was quantitated by measuring the number of tubes (**d**), as well as the tube diameter (**e**). The data in the graph is the mean ± s.e.m. of the three fields using three independent experiments. (**f**) An equivalent number of HEC1A/EMP2 endometrial tumor cells were treated for 12 h under hypoxic conditions with varying concentrations of EMP2 IgG1 or control IgG. Supernatants were collected and added to HUVEC plated on low growth factor matrigel. Capillary tube formation was measured using phase contrast microscopy after 12–24 h. The experiment was repeated three times, with the data presented as the mean ± s.e., *$P < 0.05$.

EMP2 expression directly correlated with total VEGF protein levels (Figure 4b) as well as with secreted VEGF (Figure 4c). In contrast, reduction of EMP2 resulted in undetectable levels of VEGF by western blot and low levels of secreted protein. To confirm these results, semi-quantitative RT-PCR was performed on hypoxic HEC1A/EMP2, HEC1A/V and HEC1A/RIBO cells. VEGF-A exists as multiple isoforms, which are generically referred to as VEGFxxx and result from the pre-mRNA alternative splicing of eight exons.[24] Alternative splicing of VEGF-A was initially shown to generate four different isoforms with 121, 165, 189 and 206 amino acids (VEGF121, VEGF165, VEGF189, VEGF206, respectively[25]). EMP2 levels directly increased the mRNA expression of several VEGF isoforms (VEGF165 and VEGF121), whereas a reduction in EMP2 produced reduced VEGF expression (Figure 4d). Low levels of VEGF189 mRNA was observed in HEC1A/EMP2 cells, and no expression of VEGF206 was detected in any of the cell lines.
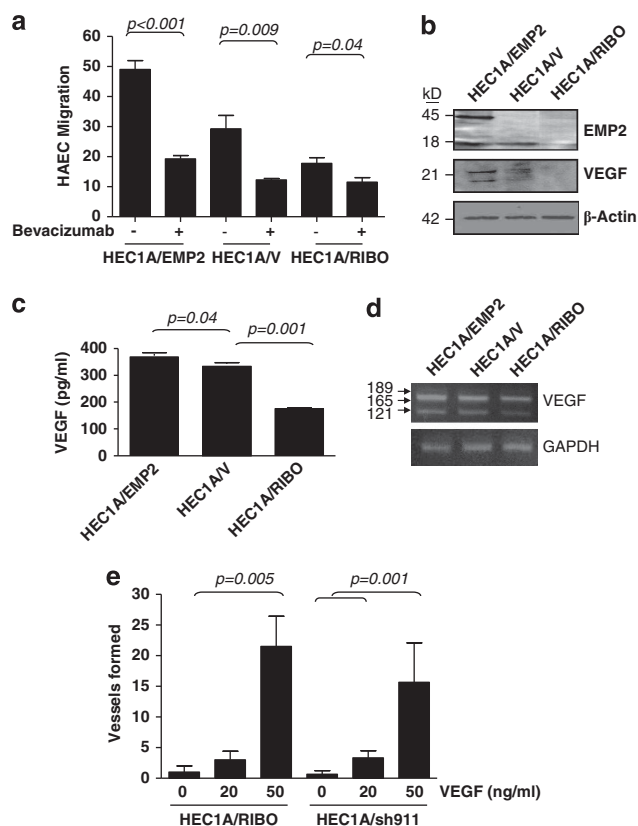
Figure 4. EMP2 regulates VEGF expression. (**a**) A Boyden chamber assay was used to determine HAEC response to cultured hypoxic tumor cell supernatants. In some experiments, the anti-VEGF antibody bevacizumab, which binds soluble VEGF was added at $10 \mu g/ml$ to the cultured supernatant. (**b**) The expression of total VEGF was measured using western blot analysis on cells placed in a 0.5% hypoxic chamber for 24 h. EMP2 expression was verified in cell lines, where its expression was either upregulated as an EMP2-GFP fusion protein (45 kDa) or reduced using an EMP2-specific ribozyme; endogenous EMP2 is 18 kDa. β-actin was used as the loading control. (**c**) Secreted VEGF from hypoxic cells was measured using an ELISA. (**d**) Semi-quantitative expression of VEGFxxx mRNA was determined using RT-PCR on hypoxic tumor cells. The experiment was repeated three times with similar results, and a representative blot is shown. GAPDH expression serves as a loading control. (**e**) Exogenous VEGF was added to cultured media obtained from hypoxic HEC1A/RIBO or HEC1A/sh911 cells at 20 or 50 ng/ml. Capillary-like tube formation was measured using phase contrast microscopy after 12–24 h. The experiment was repeated three times, with the data presented as the mean ± s.e.

In the previous experiments, we demonstrated that down-regulation of EMP2 in the tumor cell reduced capillary formation, and the motility of endothelial cells using coculture assays. This may be the result of reduced VEGF secretion by these cells or due to the expression of anti-angiogenic agents. To address this question, varying amounts of VEGF were added to the super-natants from vehicle control or cells with reduced EMP2 levels (HEC1A/RIBO or HEC1A/sh911). Exogenous VEGF was sufficient to induce HUVEC cell tube formation (Figure 4e), suggesting that the absence of VEGF contributed to the lack of tube formation.

EMP2 promotes HIF-1α expression through a Src-mediated pathway

Several oncogenic as well as growth factor-driven pathways have been shown to regulate VEGF expression. As hypoxic conditions have been shown to trigger many oncogenic signaling pathways,[26] we initially investigated if the levels of EMP2 were sufficient to alter the expression of hypoxia-induced transcription factors (HIF), specifically HIF-1α and HIF-2α. In all EMP2 HEC1A variants, no expression of HIF-2α was observed (data not shown). However, the expression of HIF-1α correlated with EMP2 expression. The highest levels of HIF-1α directly correlated with the highest concentration of EMP2 expression under hypoxic conditions (Figure 5a). Reciprocally, HEC1A/RIBO cells produced below detection levels of HIF-1α under the same conditions. To confirm that lower levels of EMP2 reduce HIF-1α expression, shRNA constructs were generated to express a vehicle control or reduce EMP2 expression (Figure 5a, right). Similar to the ribozyme, HEC1A/sh911 showed reduced HIF-1α expression compared to the vehicle control (HEC1A/shCtrl). To quantitate the difference in HIF-1α expression between the cell lines, blot intensities were analyzed using Image J. HEC1A/EMP2 cells significantly induced HIF-1α expression compared to HEC1A/V cells (Figure 5b; $P = 0.0008$). Reduction in EMP2 in HEC1A/RIBO, also showed a significant reduction in HIF-1α expression ($P = 0.02$).

HIF proteins have a number of important roles in cellular physiology that include the regulation of angiogenesis as well as metabolism.[26,27] To determine if the regulation of HIF-1α expression by EMP2 is restricted to VEGF, proteins involved with glycolysis were examined (Figure 5c). EMP2 expression directly correlated with glucose transporter-1 (GLUT1) expression, and significant differences were observed between HEC1A/EMP2 and HEC1A/sh911 ($P = 0.04$). Whereas high EMP2 also appeared to slightly increase the expression of lactate dehydrogenase A, no significant differences were observed between the cell lines (Figure 5c).

We next focused on understanding the mechanism by which EMP2 regulated HIF-1α expression. As EMP2 has been shown to promote integrin-mediated FAK and Src activation,[9,28] the contribution of FAK/Src signaling to HIF-1α expression was determined. Using common inhibitors of AKT, PI3-kinase, EGFR and Src tyrosine kinases, HEC1A/EMP2 cells were treated with these agents while in a hypoxic chamber. As shown in Figure 5b, EMP2-induced overexpression of HIF-1α was reversed using Src inhibitors PP2 and dasatinib, respectively. Both erlotinib and Ly294002, a PI3-kinase inhibitor, also produced a similar effect, suggesting an overlap in signaling with EMP2. In contrast, AKTi did not alter HIF-1α expression in these cells (Figure 5d).

To validate the effects of FAK signaling on HIF-1α expression, HEC1A/EMP2 and HEC1A/V cells were transiently transfected with scrambled or FAK siRNA (Figure 5e). After 24 h in a hypoxic chamber, HIF-1α expression was determined. Reduction in FAK expression significantly diminished HIF-1α expression in both cell lines (Figure 5e, right). A $68 \pm 3\%$ reduction in FAK expression decreased HIF-1α expression by $48 \pm 3\%$ in HEC1A/EMP2 cells. A similar reduction was observed in HEC1A/V cells where decreasing FAK expression by $51 \pm 5\%$ produced an $82 \pm 8\%$ drop in HIF-1α expression.

DISCUSSION

EMP2 is a novel oncogene upregulated in a number of cancers in women. In endometrial cancer, for example, EMP2 expression promotes endometrial cancer growth in vivo,[9] and to date, it is the only biomarker identified to predict endometrial cancer prognosis and survival.[11,14] Given its expression profile and importance in disease pathogenesis, we previously generated a recombinant antibody fragment (diabody) to EMP2, and have shown that it induces necrosis in vivo.[15] Since then, we have generated an EMP2 IgG1 with similar in vitro and in vivo properties (Fu and Wadehra, submitted), and have started to characterize its mechanism of action in vitro and in vivo.

Using endometrial cancer cells, EMP2 IgG1 treatment signifi-cantly reduced tumor load with a net improvement in survival.
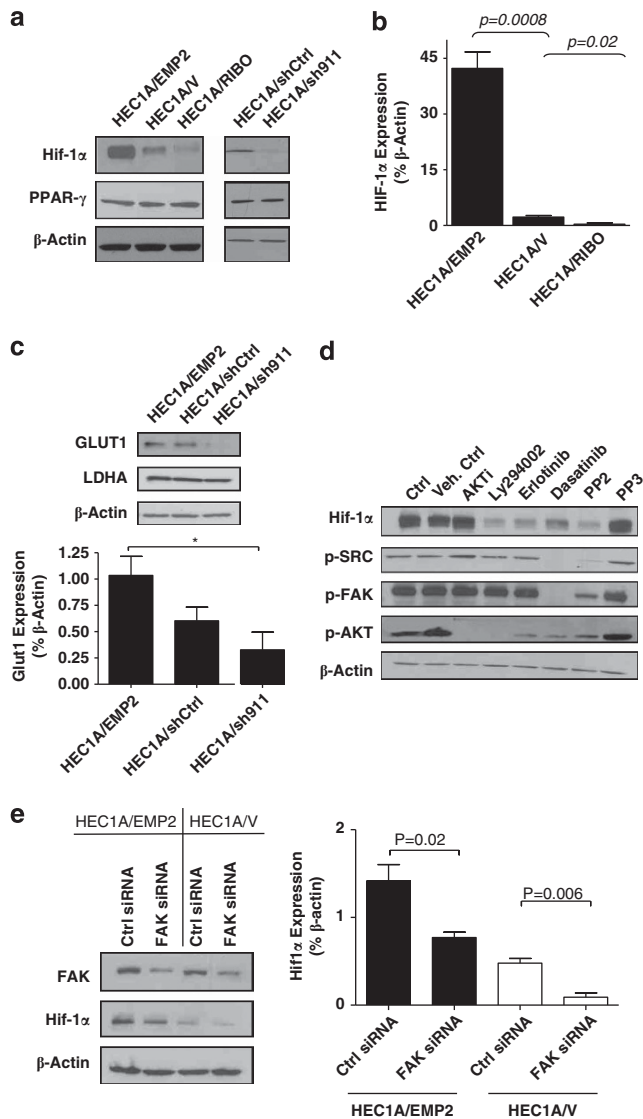
Figure 5. EMP2 regulates VEGF through FAK-Src mediated control of HIF-1α. (**a**) EMP2 expression was downregulated using either a ribozyme (HEC1A/RIBO) or shRNA lentiviral constructs (HEC1A/sh911). Appropriate vehicle control cells were included. To determine the mechanism for VEGF regulation, HIF-1α and PPARγ expression were determined using western blot analysis on hypoxic cells. β-actin expression serve as the loading control. (**b**) Quantitation of HIF-1α expression in HEC1A/EMP2, HEC1A/V and HEC1A/RIBO relative to β-actin from three independent experiments. The data represents the mean ± s.e. (**c**) To determine if other HIF-1α regulated proteins were altered by EMP2 levels, hypoxic lysates were probed for GLUT1 and lactate dehydrogenase A expression. Below, quantitation of GLUT1 expression from three independent blots. *$P = 0.04$. (**d**) HEC1A/EMP2 cells were incubated with the PP2, PP3, Dasatinib, Erlotinib, Ly294002, AKTi VIII, a DMSO vehicle control or a media control for 24 h in a 0.5% hypoxic chamber. Cells were probed for the protein expression of HIF-1α, p-FAK, p-SRC, p-AKT and β-actin. (**e**) HEC1A/EMP2 or HEC1A/V cells were transiently transfected with FAK siRNA or a scrambled control. Lysates were created from cells placed in a hypoxic chamber for 24 h and analyzed using SDS-PAGE/western blot analysis. Blots were probed for HIF-1α, total FAK and β-actin levels. Right, quantitation of HIF-1α levels relative to β-actin following transfection of cells with a FAK or scrambled siRNA.

Within tumors, significant necrosis was observed. To understand its mechanism of action, we generated cells with reduced EMP2 expression using ribozymes or specific shRNA constructs, which

revealed a similar histology. In particular, severe necrosis was observed in tumors with reduced EMP2. Although we had previously observed increased cell death *in vitro* after exposure to antibodies that recognize EMP2, the necrotic response and the studies reported in this paper link EMP2 with control of VEGF expression through HIF-1α. Notably, HEC1A cells that were genetically modified for EMP2 expression showed a positive correlation between EMP2 levels and tumor vascularity. Cell supernatants from cells that were genetically modified for EMP2 expression showed a positive correlation between EMP2 levels, and endothelial cell migration and tube formation of two independent endothelial types under hypoxic conditions. Levels of VEGF and HIF-1α were concordant also with expression levels of EMP2, and blockade of EMP2 using an anti-EMP2 antibody showed a dose-dependent decrease in vascularization.

Whereas additional investigations will be needed to identify how EMP2 controls HIF-1α expression, its effects do not appear to be limited to angiogenesis. As HIF-1 also regulates metabolism,[26] the significant alteration in GLUT1 expression suggests that EMP2 upregulation may have an adaptive role in promoting and establishing endometrial cancer tumorigenesis. Whereas it is known that several oncogenes stimulate angiogenesis,[29] all of the studies presented here point to a significant effect on HIF-1α and VEGF through control of EMP2 expression, and suggest the mechanism for the clinical association of high EMP2 expression with aggressive, more advanced tumors.[12,14] Our results also suggest that EMP2 activates HIF-1α in a hypoxic environment through a FAK-Src-dependent mechanism. Although a similar trend in HUVEC migration and wound closure was observed under normoxic conditions, the differences were not statistically significant (data not shown). Under hypoxic conditions, the control of angiogenesis by EMP2 appears to be independent of AKT activation, suggesting that EMP2 and AKT regulate separate pathways. This is consistent with published reports that AKT signaling is neither required for HIF-1α expression nor stabilization under hypoxic conditions.[30] Although AKT inhibitors did not suppress EMP2 mediated HIF-1α activation, erlotinib and Ly294002, the phosphatidylinositol 3-kinase (PI3K) inhibitor, were sufficient to inhibit its expression. This is not unexpected as EGFR neutralizing antibodies have been shown to have potent antitumor cytotoxic-like effects *in vivo* (where angiogenesis is required) but not *in vitro* (where it is not).[23] This would also suggest that in endometrial cancer cells, similar to what has been observed in other tumor models, EGFR stimulates HIF-1α activation via a PI3K-mediated but AKT independent manner.[31,32] Is there overlap between EMP2 and EGFR signaling? We hypothesize that there is some overlap. Previous studies have shown that EMP2 promotes integrin expression and activation.[33,34] As both integrins and EGFR can signal through MAP kinase activation, we predict this may be a common thread between the two pathways.[31,35]

The data presented here suggest that anti-EMP2 therapy in the disease has a combination of effects (direct toxicity and indirect regulation of VEGF) that supports its therapeutic potential. This is particularly important, given that the promise of anti-angiogenic agents has been incomplete.[36] Initial clinical successes, prolonged overall and progression-free survival, using anti-VEGF antibody therapy in colorectal cancer prompted more widespread clinical use of these agents. However, these promising results were not replicated in multiple tumor types including metastatic breast cancer, resulting in withdrawal of FDA approval for its use in this tumor type. Indeed, some tumors escape VEGF inhibition through both hypoxia-dependent and hypoxia-independent manners. In addition, VEGF blockade may lead to proinflammatory molecules that might enhance tumor progression.[37] Could a similar effect be observed with longer anti-EMP2 treatment? Although our results are promising, additional investigations are required to further to define the long-term consequences of anti-EMP2 treatment.

MATERIALS AND METHODS

Cell lines

The human endometrial adenocarcinoma cell line HEC1A (HTB112, ATCC, Manassas, VA, USA) was cultured in McCoys media supplemented with 10% fetal calf serum at 37 °C in a humidified 5% CO_2 incubator. Cell lines were used within 2 months after resuscitation of frozen aliquots, and were authenticated based on viability, recovery, growth, morphology and isoenzymology by the supplier. Stably transfected HEC1A cells containing a human EMP2-GFP fusion protein, control GFP or EMP2-specific ribozyme have been previously described.[33,38] Stably infected HEC1A cells containing a non-targeting shRNA control (HEC1A/shCtrl) or EMP2-specific shRNA (HEC1A/sh911) (911; TRCN0000322911) in pLKO.1-puro were generated as per manufacturer's instructions (Sigma-Aldrich, St Louis, MO, USA).

Human umbilical vein endothelial cells (HUVEC) from passage 2 were purchased from BD Biosciences (San Diego, CA, USA), and used between passages 3 and 7 for all experiments. Primary HAECs (gift from Dr J Berliner, UCLA) were also utilized. All endothelial cells were grown in complete MCDB-131 complete media (VEC Technologies, Renesselaer, NY, USA).

Preparation of xenografts

Ethical treatment of animals statement: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Research Committee at the University of California, Los Angeles CA, USA. All efforts were made to minimize animal suffering.

Four- to six-week-old nude BALB/c female mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and maintained at the University of California, Los Angeles, CA, USA. Animals were inoculated subcutaneously with 1×10^6 HEC1A/EMP2, HEC1A/V or HEC1A/RIBO cells into the right and left shoulder flanks, respectively. Tumors were measured using calipers, and the volume calculated with the formula: length × width^2/2. Six mice were used per group. At day 30, tumors were excised, fixed in formalin, and then processed for hematoxylin and eosin staining by the Tissue Procurement Laboratory at UCLA or by Masson's trichrome stain (Dako, Carpinteria, CA, USA).

In some experiments, 1×10^6 HEC1A/EMP2 cells were suspended in 5% matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and injected subcutaneously into the shoulder of female athymic mice. When tumors approached 4 mm^2, systemic treatments with 10 mg/kg dose of anti-EMP2 IgG1 or control IgG (Sigma-Aldrich) were administered weekly via an intraperitoneal route. Tumor size was monitored, and mice euthanized once tumors approached 1.5 cm in diameter, mice appeared moribund or showed a 10% reduction in weight, or tumors became ulcerated. Tumors were isolated, fixed and processed for hematoxylin and eosin staining as previously described.[14]

Cytochemistry and immunohistochemistry

Samples were analyzed to quantitate and identify tumor vasculature. Masson's trichrome staining was performed according to the manufacturer's instructions (Dako). Samples were deparaffinized and dehydrated in alcohol. In some experiments, after blocking in 1% normal goat serum, samples were incubated with 1:50 dilution of FITC-labeled *L. esculentum* lectin (1 mg/ml in 0.9% NaCl; Vector Laboratories, Burlingame, CA, USA). Samples were then counterstained with DAPI and mounted. To quantitate CD34 expression, samples were deparaffinized and then incubated at 95 °C for 20 min in 0.1 M citrate, pH 6.0. Rat anti-CD34 (Abcam, Cambridge, MA, USA) was used at a dilution of 1:25 as previously described[39], followed by visualization using the Vector ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The numbers of CD34-positive blood vessels in six high-power fields (× 200) were counted and averaged.

Capillary tube formation

Capillary-like tube formation was performed as previously described.[40] Briefly, coverslips were coated with reduced growth factor basement membrane (Geltrex; Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C for 30 min to promote jelling. 5×10^5 HUVECs were resuspended in cultured media from HEC1A/EMP2, HEC1A/V or HEC1A/RIBO cells. Conditioned media was typically prepared as follows: cells were grown in a bag to mimic hypoxic conditions for up to 72 h or placed in a 0.5% hypoxic chamber (BioSpherix, Lacona, NY, USA) for up to 24 h. The cultured media was then collected and centrifuged to remove cell debris. In some

experiments, HEC1A or HEC1A/EMP2 cells were treated with 100 µg/ml of the full-length EMP2 IgG1 for 24 h under hypoxic conditions for 12–15 h. In other experiments, recombinant human VEGF (Sigma-Aldrich) was used as an inducer of capillary tube formation from 0–50 ng/ml on HUVEC cells. After 18 h, cells were stained with calcein AM and analyzed using an Olympus BX51 light microscope. Tubes were counted using a 10 × objective connected to a DP72 digital camera. Three random fields were measured at each culture condition. Each experiment was repeated at least three times.

Migration assays

HUVEC and HAEC migration assays were conducted in Boyden chambers as previously reported (2). Conditioned media was prepared as described above. In some experiments, cultured supernatants from tumor cells were treated with bevacizumab (Genentech BioOncology, South San Francisco, CA, USA). Bevacizumab was obtained from the pharmacy at University of California, Davis, CA, USA. Each condition was assayed in triplicate wells. 'Scratch' wound closure assays were performed by creating a confluent monolayer of HUVEC cells. Using the tip of a Pasteur pipet, a scratch was created. Conditioned media from HEC1A/EMP2, HEC1A/V or HEC1A/RIBO cells were added to the wells. Three random measurements for each of the three wounds were measured for each test condition. The experiment was repeated three times.

Semi-quantitative RT-PCR

For RT-PCR analysis, total cellular RNAs were isolated using RNeasy mini kit (Qiagen, Valencia, CA, USA). In all conditions, 1 µg of total RNA was reversed transcribed using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). For VEGF amplification including all four splice variants, the PCR conditions and primers were utilized as previously described.[41] Amplification of a GAPDH cDNA fragment was performed in a separate PCR reaction as described.[42] PCR products were run on a 2% agarose gel and were visualized by ethidium bromide staining.

FAK siRNA

In some experiments, cells were transiently transfected with FAK siRNA or a scrambled control as previously described.[8] Briefly, HEC1A/EMP2 or HEC1A/V cells were transiently transfected with 75 pmol FAK siRNA (L-003164-00, ThermoScientific, Lafayette, CO, USA) or a scrambled control (D-001206-13-05, ThermoScientific). After 6 h, cells were placed in a 0.5% hypoxic chamber for 24 h and then processed for western blot analysis as detailed below.

Western blot analysis

Cells were lysed in Laemmli buffer. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare Biosciences, Piscataway, NJ, USA), and stained with Ponceau S (Sigma-Aldrich) to determine transfer efficiency. Membranes were blocked with 10% low-fat milk in PBS containing 0.1% Tween 20 and probed with EMP2 antisera (1:1000), anti-VEGF (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-HIF-1α (1:800; BD Biosciences), anti-PPAR-γ (Santa Cruz Biotech), anti-[416] p-Src (Cell Signaling, Danvers, MA, USA), anti-p-AKT (Cell Signaling), anti-[576/577]p-FAK (1:500; BD Biosciences) or β-actin (Sigma-Aldrich). Protein bands were visualized using a horseradish peroxidase-labeled secondary antibody (BD Biosciences; Southern Biotechnology Associates, Birmingham, AL, USA) followed by chemiluminescence (ECL; GE Healthcare Biosciences). Band intensities were quantified using the NIH program Image J as above. To account for loading variability β-actin was used to normalize each sample. At least three independent experiments were performed and, where indicated, the results were evaluated for statistical significance using a Student's *t*-test (unpaired, one-tail). A level of $P < 0.05$ was considered to be statistically significant.

In some experiments, inhibitors were added to determine the contribution of specific pathways to HIF-1α expression. HEC1A/EMP2 cells were treated with 10 µM of the FAK-Src small molecule inhibitor PP2 or the small molecule control PP3,[8] 5 µM of Akt inhibitor VIII (Barnett *et al*.[16] Calbiochem, San Diego, CA, USA), 50 µM of the PI3 kinase inhibitor Ly294002 (Cell Signaling), the EGFR inhibitor Erlotinib (Wang *et al*.[17] 10 µM, Genentech) or the Src family tyrosine kinase inhibitor Dasatinib (Lu *et al*.[43] 10 µM, Bristol-Myers Squibb). Efficacy of the inhibitors was tested at the manufacturer's recommended dosage, and potential toxicity was

measured using trypan-blue exclusion. Samples were harvested and probed by SDS–PAGE/western blot analysis as above.

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