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ORIGINAL ARTICLE

HEXIM1 modulates vascular endothelial growth factor expression and function in breast epithelial cells and mammary gland

N Ogba[1], YQ Doughman[2], LJ Chaplin[1], Y Hu[1], M Gargesha[3], M Watanabe[2] and MM Montano[1]

[1]*Department of Pharmacology, Rainbow Babies and Children's Hospital, Cleveland, OH, USA;* [2]*Department of Pediatrics, Rainbow Babies and Children's Hospital, Cleveland, OH, USA and* [3]*Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH, USA*

Recently, we found that mutation of the C-terminus of transcription factor hexamethylene bisacetamide-inducible protein 1 (HEXIM1) in mice leads to abnormalities in cardiovascular development because of aberrant vascular endothelial growth factor (VEGF) expression. HEXIM1 regulation of some genes has also been shown to be positive transcription elongation factor b (P-TEFb) dependent. However, it is not known whether HEXIM1 regulates VEGF in the mammary gland. We demonstrate that HEXIM1 regulates estrogen-induced VEGF transcription through inhibition of estrogen receptor-α recruitment to the *VEGF* promoter in a P-TEFb-independent manner in MCF-7 cells. Under hypoxic conditions, HEXIM1 inhibits estrogen-induced hypoxia-inducible factor-1 alpha (HIF-1α) protein expression and recruitment of HIF-1α to the hypoxia-response element in the *VEGF* promoter. In the mouse mammary gland, increased HEXIM1 expression decreased estrogen-driven VEGF and HIF-1α expression. Conversely, a mutation in the C-terminus of HEXIM1 ($HEXIM1_{1-312}$) led to increased VEGF and HIF-1α expression and vascularization in mammary glands of heterozygous $HEXIM1_{1-312}$ mice when compared with their wild-type littermates. In addition, $HEXIM1_{1-312}$ mice have a higher incidence of carcinogen-induced mammary tumors with increased vascularization, suggesting an inhibitory role for HEXIM1 during angiogenesis. Taken together, our data provide evidence to suggest a novel role for HEXIM1 in cancer progression.
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Introduction

Estrogens have a significant role in the etiology and progression of breast cancers and mediate their actions through estrogen receptors (ERs), ER-α and ER-β, nuclear steroid receptors that regulate transcription either directly by binding to estrogen-response elements of target genes or indirectly through protein–protein interactions with other transcription factors (Kushner *et al*., 2000; Deroo and Korach, 2006). Another factor known to have an important role in tumor progression is the vascular endothelial growth factor (VEGF) (Ferrara *et al*., 2003). VEGF mediates its effects through its receptors, VEGFR1 and VEGFR2, and regulates angiogenesis in both physiological and pathological processes, including tumor-associated angiogenesis (Ferrara *et al*., 2003). VEGF is highly expressed in many breast tumors and VEGF represents a major target for tumor therapy (Rugo, 2004; Ellis and Hicklin, 2008).

Several studies have shown that estrogens modulate VEGF expression in breast and uterus tissues and in breast cancer cell lines (Hyder, 2006). The *VEGF* gene is also known to be estrogen responsive and have ER-α-regulatory components (Stoner *et al*., 2004; Kazi *et al*., 2005). In addition, many tumors coexpress ER-α and VEGF (Kimbro and Simons, 2006). VEGF expression is also induced by hypoxia (Ferrara *et al*., 2003; Kimbro and Simons, 2006). The regulation of VEGF expression by hypoxia occurs because of the stabilization of hypoxia-inducible factor-1 alpha (HIF-1α) protein levels, which interacts with its constitutively expressed binding partner, HIF-1β, and the heterodimer binds the hypoxia-response element (HRE) in the *VEGF* promoter to induce its expression (Kimbro and Simons, 2006). HIF-1α has been shown to be a positive regulator of tumor progression and high levels of HIF-1α expression occur in ER-α-positive and negative breast cancers (Bos *et al*., 2004; Kimbro and Simons, 2006; Liao *et al*., 2007). In addition, estrogens have been shown to induce HIF-1α expression and enhance the recruitment of HIF-1α to the *VEGF* promoter in the rat uterus and in endometrial cancer cells (Kazi *et al*., 2005; Molitoris *et al*., 2009).

We have shown that increased expression of hexamethylene bisacetamide-inducible protein 1 (HEXIM1) inhibits breast cell growth (Wittmann *et al*., 2003). We also demonstrated that HEXIM1 interacts with ER-α and decreases its transcriptional activity (Wittmann *et al*., 2005). Several studies have also shown that HEXIM1 interacts with the positive transcription elongation factor b (P-TEFb) through cyclin T1 and

Correspondence: Dr MM Montano, Department of Pharmacology, Case Western Reserve University, HG Wood Building W305, 2109 Adelbert Road, Cleveland, OH 44106, USA.
E-mail: mxm126@case.edu
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inhibits its activity (Zhou and Yik, 2006). P-TEFb is a complex of cyclin T1 and cyclin-dependent kinase 9 that phosphorylates the carboxy terminal domain of RNA polymerase II (RNAP II) to promote productive phases of transcription (Sims *et al*., 2004). We found that estrogen enhances P-TEFb activity and increases the recruitment of P-TEFb to some ER-α target genes (Ogba *et al*., 2008). HEXIM1 regulation of these genes involves inhibiting P-TEFb activity and recruitment to the ER-α target genes (Ogba *et al*., 2008). In addition, a recent study from our laboratory uncovered a novel role of HEXIM1 as a regulator of VEGF during heart and vascular development using a mouse model with a C-terminus mutation in HEXIM1 (Montano *et al*., 2008).

Although it is known that estrogens can induce VEGF expression through ER-α in breast cancer cells, it is not known whether this regulation is dependent on P-TEFb. In addition, it is not known whether HEXIM1 regulates VEGF expression in breast cancer cells, the mammary gland and in mammary tumors. In this study, we demonstrate that in breast cells, HEXIM1 regulates VEGF expression by its effect on transcription factors ER-α and HIF-1α, suggesting an important role for HEXIM1 in mammary tumorigenesis.

Results

Increase in HEXIM1 expression inhibits estrogen-induced VEGF expression in breast cancer cells

To determine the effect of HEXIM1 on VEGF expression, MCF-7 breast cancer cells, which are ER-α-positive, were transfected with empty vector or pCMV-Tag2B-HEXIM1 and treated with ethanol (vehicle) or increasing concentrations of 17-β estradiol (E_2). There was a significant increase in VEGF mRNA expression in cells treated with 1 and 10 nM E_2 (Figure 1a, lanes 2 and 3). Although the E_2-induced increases in VEGF mRNA were modest (~1.5-fold increase relative to vehicle-treated cells), previous reports using breast cancer cells cultured *in vitro* have observed E_2-induced increases in VEGF mRNA within a similar range (Maity *et al*., 2001; Higgins *et al*., 2006). The MCF-7 cells were adequately responsive to E_2, as there were significant increases in other ER target genes, including trefoil factor 1 (TFF1 or pS2) and progesterone receptor (Supplementary Figure 1). Increased HEXIM1 expression in MCF-7 cells inhibited E_2-induced increases in VEGF mRNA expression (Figure 1a, lanes 5 and 6). Increased HEXIM1 expression also inhibited E_2-induced pS2 mRNA expression but did not affect E_2-induced progesterone receptor mRNA expression as previously reported (Supplementary Figure 1) (Ogba *et al*., 2008).

From previous studies, we know that HEXIM1 inhibits E_2/ER-α transcriptional activity in the context of some ER-α target genes (Wittmann *et al*., 2005; Ogba *et al*., 2008). In ER-α-negative MDA-MB-231 cells, we found no change in VEGF mRNA expression in response to estrogen treatment or increased HEXIM1 expression (Figure 1b), suggesting that the effect of increased HEXIM1 expression on VEGF mRNA expression requires E_2/ER-α. To further test this hypothesis, we transfected MDA-MB-231 cells with control vector or pCMV-Tag2B-ER-α and treated cells with vehicle or E_2. MDA-MB-231 cells are typically not E_2 responsive except in some cases of ER-α or ER-β-overexpression (Buteau-Lozano *et al*., 2002), but in our hands, we found that VEGF mRNA was slightly, but significantly responsive to E_2 in ER-α-expressing MDA-MB-231 cells (Supplementary Figure 2, lane 3) and increased HEXIM1 expression inhibited the E_2-induced increase in VEGF mRNA expression in these cells (Supplementary Figure 2, lane 5).

To determine whether increased HEXIM1 expression exerted any effect on estrogen-induced secreted VEGF protein, we examined changes in the levels of secreted VEGF protein in the media of cultured MCF-7 cells and found that increased HEXIM1 expression decreased E_2-induced secretion of VEGF protein from MCF-7 cells (Figure 1c). Taken together, these data suggest that HEXIM1 regulation of VEGF expression requires E_2/ER-α.

Increased HEXIM1 expression inhibits the recruitment of E_2/ER-α to the VEGF *promoter*

Previous studies have determined that the *VEGF* promoter contains estrogen-responsive Sp1-binding sites and GC-rich motifs that contribute to E_2/ER-α-driven VEGF transactivation (Stoner *et al*., 2004; Kazi *et al*., 2005). To determine the effect of HEXIM1 on E_2/ER-α-regulated VEGF transcription, we carried out chromatin immunoprecipitation (ChIP) assays to examine changes in the recruitment of ER-α to a region in the *VEGF* promoter proximal to GC-rich/Sp1-binding elements. We found that increased HEXIM1 expression led to an increase in HEXIM1 occupancy at the *VEGF* promoter that did not seem to be E_2 dependent (Figure 1e). This increased occupancy of HEXIM1 led to a decrease in the recruitment of E_2/ER-α and RNAP II to the *VEGF* promoter (Figure 1e and Supplementary Figure 3a). As a control, we examined the recruitment of ER-α to a region in the *VEGF* promoter that does not contain GC-rich/Sp1 sites (see control region in Figure 1d) and did not observe any recruitment to this region (Supplementary Figure 3b).

Previous studies from our laboratory have determined that HEXIM1 inhibits E_2-driven transcription of some ER-α target genes in a P-TEFb-dependent manner (Wittmann *et al*., 2005; Ogba *et al*., 2008). To determine whether P-TEFb is involved in E_2/ER-α-driven VEGF transcription, we immunoprecipitated cyclin T1 in the ChIP assays and found that cyclin T1 recruitment was not enhanced by E_2 treatment and increased HEXIM1 expression did not significantly affect its occupancy at the GC-rich/Sp1 region in the *VEGF* promoter (Figure 1e and Supplementary Figure 3a). We also examined the recruitment of cyclin T1 to the *VEGF* promoter control region

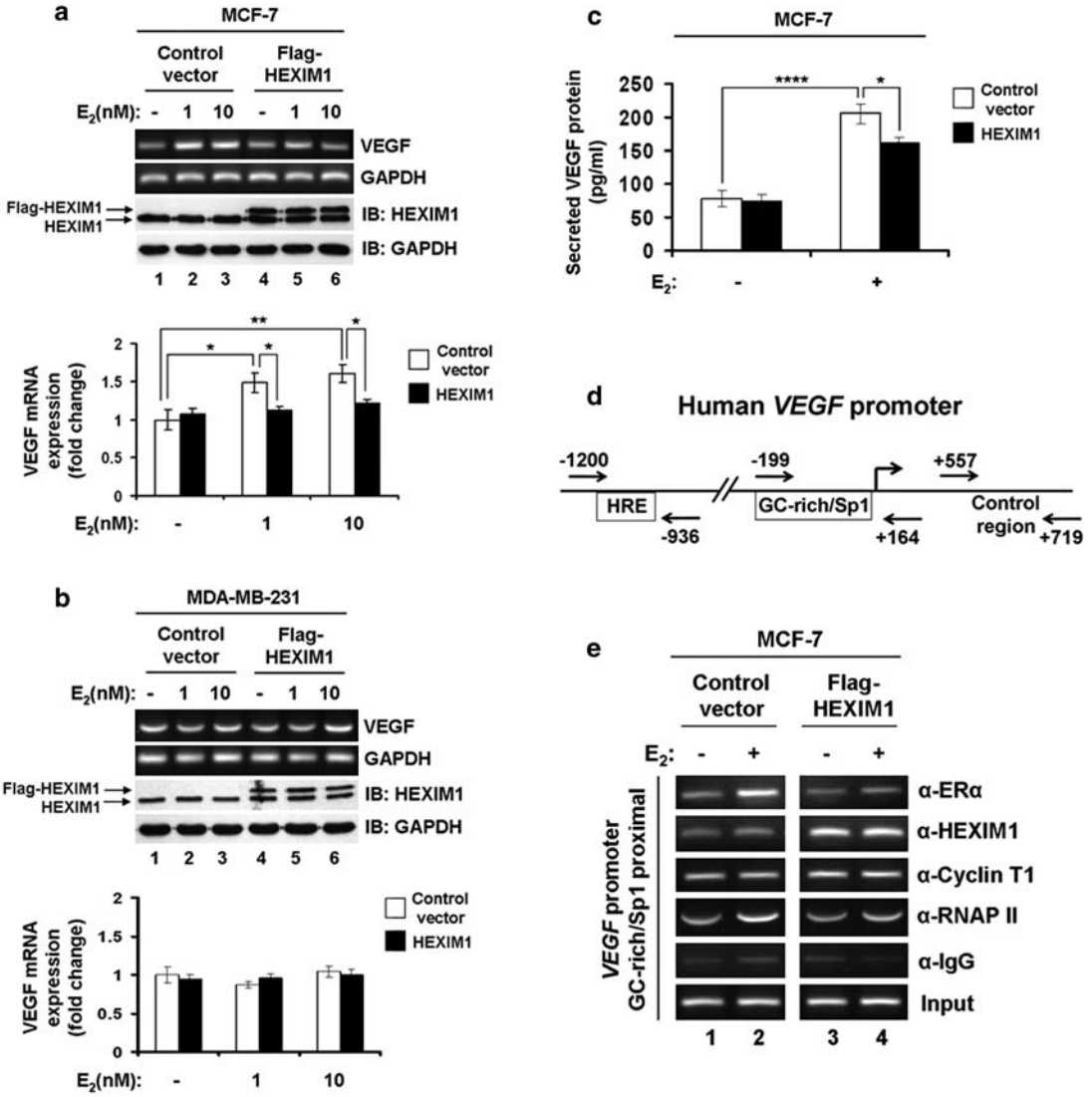

Figure 1 Increased HEXIM1 expression inhibits E_2-induced transcription of VEGF by ER-α in breast cancer cells. (**a**) MCF-7 cells were transfected with pCMV-Tag2B-HEXIM1 (Flag-HEXIM1 expression vector) or control vector and treated with ethanol (vehicle) or 1 or 10 nM 17-β estradiol (E_2) for 4 h. Graph shows fold change of VEGF mRNA expression levels measured by reverse transcriptase PCR (RT–PCR). Data represent mean ± s.e.m. from four independent experiments carried out in duplicate; '*' and '**' indicate statistical significance ($P<0.05$ and $P<0.005$, respectively). (**b**) MDA-MB-231 cells were transfected with Flag-HEXIM1 expression vector or control vector and treated with ethanol (vehicle) or 1 or 10 nM E_2 for 4 h. Graph shows fold change of VEGF mRNA expression levels measured by RT–PCR. Data represent mean ± s.e.m. from three independent experiments carried out in duplicate. (**c**) MCF-7 cells were transfected with Flag-HEXIM1 expression vector or control vector and treated with ethanol or 10 nM E_2 for 12 h. Secreted VEGF protein levels were measured by enzyme-linked immunosorbent assay. Data represent mean ± s.e.m. from four independent experiments assayed in duplicate; '*' and '****' indicate statistical significance ($P<0.05$ and $P<0.00005$, respectively). (**d**) Primers used in ChIP assays are directed at regions indicated for *VEGF* promoter. (**e**) MCF-7 cells were transfected with Flag-HEXIM1 expression vector or control vector and treated with ethanol or 100 nM E_2 for 45 min. Results show ChIP analyses of lysates immunoprecipitated with ER-α, HEXIM1, cyclin T1, RNA polymerase II (RNAP II) and rabbit immunoglobulin (IgG) antibodies. PCR amplification of the GC-rich/Sp1 proximal fragment in the *VEGF* promoter (Figure 1d) was carried out and graph shows quantification of PCR products as indicated. Data represent mean ± s.e.m. from three independent experiments.

described earlier and found that cyclin T1 was also not recruited to this region (Supplementary Figure 3b). Although it is possible that E_2 modulates cyclin T1 occupancy at other regions in the *VEGF* gene, these data suggest that P-TEFb recruitment to the GC-rich/Sp1 region of the *VEGF* promoter is not dependent on E_2 and not significantly affected by HEXIM1.

Under hypoxia, HEXIM1 inhibition of VEGF expression correlates with a decrease in E_2-induced HIF-1α expression

Low oxygen tension is another positive regulator of VEGF expression (Kimbro and Simons, 2006). To determine the effect of HEXIM1 on hypoxia-induced VEGF expression in the presence or absence of E_2, we transfected MCF-7 cells with control vector or

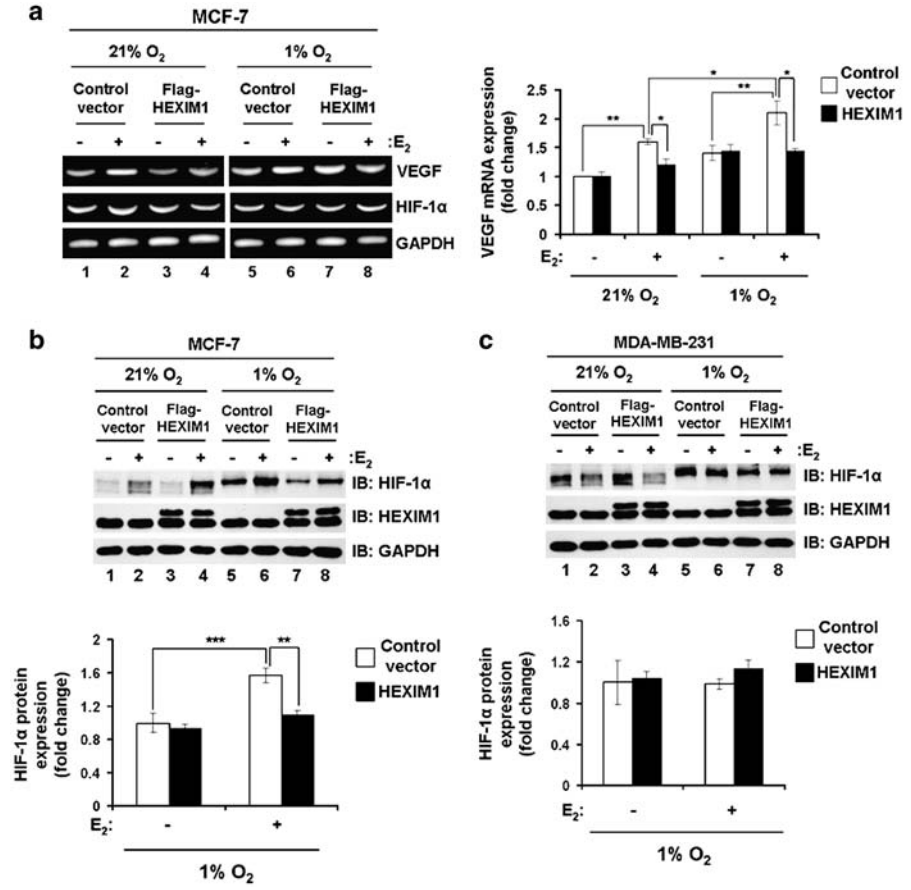

Figure 2 Increased HEXIM1 expression inhibits E_2-induced VEGF mRNA expression under hypoxia that correlates with a decrease in E_2-induced HIF-1α protein expression. (**a**) MCF-7 cells were transfected with Flag-HEXIM1 expression vector or control vector, treated with ethanol or 10 nM E_2 and grown under high oxygen (21% O_2) or low oxygen (1% O_2) conditions for 12 h. Results show fold change of VEGF mRNA expression levels measured by RT–PCR. Data represent mean ± s.e.m. from three independent experiments carried out in duplicate; '*' and '**' indicate statistical significance ($P<0.05$ and $P<0.005$, respectively). (**b**) MCF-7 cells were transfected with Flag-HEXIM1 expression vector or control vector, treated with ethanol or 10 nM E_2 and grown under 21% or 1% O_2 conditions as indicated for at least 12 h. Western blot analyses show changes in HIF-1α protein expression and protein expression of HEXIM1 and GAPDH (loading control). Data represent mean ± s.e.m. from four independent experiments carried out in duplicate; '**' and '***' represent statistical significance ($P<0.005$ and 0.0005, respectively). (**c**) MDA-MB-231 cells were transfected with Flag-HEXIM1 expression vector or control vector, treated with ethanol or 10 nM E_2 and grown under 21% or 1% O_2 conditions as indicated for at least 12 h. Western blot analyses show changes in HIF-1α protein expression and protein expression of HEXIM1 and GAPDH (loading control). Data represent mean ± s.e.m. from three independent experiments carried out in duplicate.

Flag-HEXIM1 expression vector and subjected the cells to either high oxygen (21% O_2) or low oxygen (1% O_2) conditions. We found that increased HEXIM1 expression inhibited E_2-induced increases in VEGF mRNA expression under both 21 and 1% O_2 conditions (Figure 2a). However, under 1% O_2 conditions alone, HEXIM1 did not inhibit VEGF mRNA expression (Figure 2a, compare lanes 5 and 7), suggesting that the effect of HEXIM1 on VEGF expression may involve the modulation of E_2 and hypoxia in concert.

Studies have shown that both estrogens and hypoxia regulate VEGF expression in breast cancer cells (Maity *et al*., 2001; Seifeddine *et al*., 2007). We found that E_2 and hypoxia (1% O_2) induced a slightly higher fold increase in VEGF mRNA expression when compared with E_2 under 21% O_2 conditions that is statistically significant ($P<0.05$) (Figure 2a). However, there was no change in HIF-1α mRNA expression in response to E_2 treatment or increased HEXIM1 expression under 21 and 1% O_2 conditions (Figure 2a), suggesting that the effect of HEXIM1 on HIF-1α is probably through post-translational regulation of HIF-1α. Western blot analyses showed that E_2 induced an increase in HIF-1α protein expression (Figure 2b). However, increased HEXIM1 expression inhibited E_2-induced increases in HIF-1α protein expression under hypoxia (Figure 2b, compare lanes 6 and 8). In MDA-MB-231 cells, we found that E_2 did not enhance HIF-1α protein expression and increased HEXIM1 expression did not affect HIF-1α protein expression (Figure 2c, see lanes 5–8). These data suggest that E_2/ER-α regulates HIF-1α protein expression to enhance VEGF expression in breast cancer cells and that HEXIM1 modulates E_2/ER-α-regulated HIF-1α protein expression under low oxygen levels.

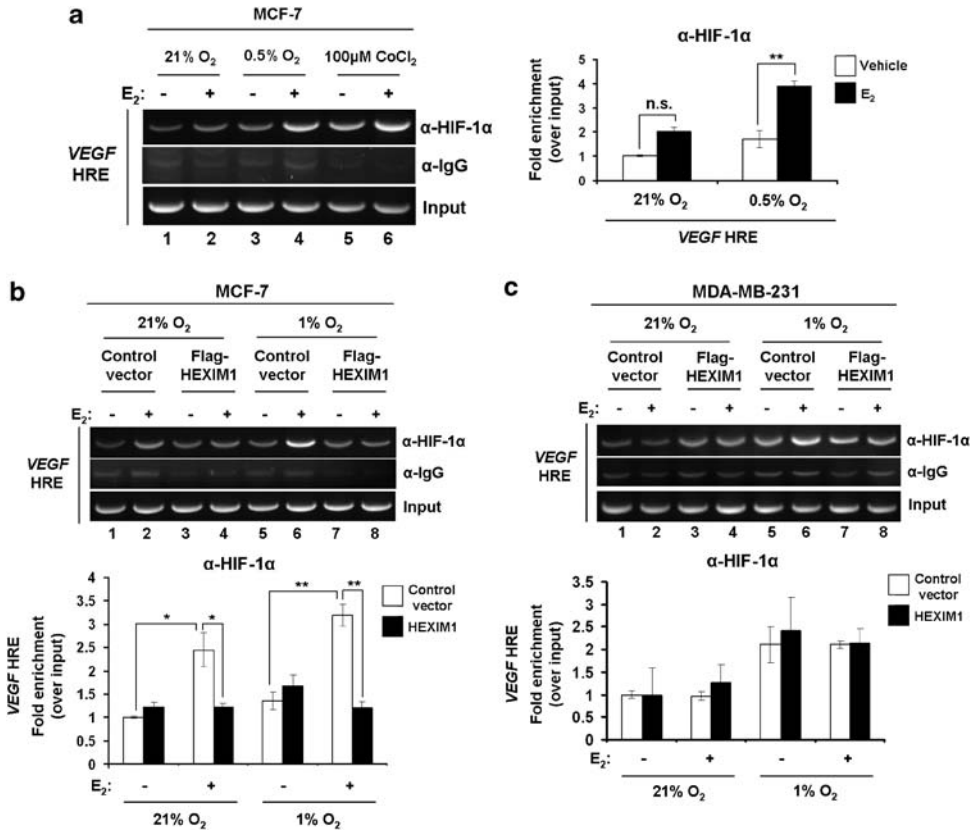

Figure 3 Increased HEXIM1 expression inhibits E_2-induced recruitment of HIF-1α to *VEGF* hypoxia-response element (HRE). (**a**) MCF-7 cells were treated with ethanol or 100 nM E_2 and subjected to high (21%) or low (0.5%) oxygen conditions as indicated or treated with 100 μM cobaltous chloride ($CoCl_2$) for 6 h. Results show ChIP analyses of lysates immunoprecipitated with HIF-1α and rabbit immunoglobulin (IgG) antibodies and DNA fragments were analyzed by PCR primers specific for HRE in the *VEGF* promoter (region indicated in Figure 1d). Data represent mean ± s.e.m. from three independent experiments; '**' indicates statistical significance ($P<0.005$). (**b**) MCF-7 cells were transfected with Flag-HEXIM1 expression vector or control vector, treated with ethanol or 100 nM E_2 and subjected to 21 or 1% O_2 conditions as indicated for 16 h. Results show ChIP analyses of HIF-1α and rabbit IgG immunoprecipitates with PCR amplification of fragment containing *VEGF* HRE. Graphs show quantification of HIF-1α immunoprecipitates and data represent mean ± s.e.m. from at least four independent experiments; '*' and '**' indicate statistical significance ($P<0.05$ and $P<0.005$, respectively). (**c**) MDA-MB-231 cells were transfected with Flag-HEXIM1 or control vector, treated with ethanol or 100 nM E_2 and subjected to 21 or 1% O_2 conditions as indicated for 16 h. Results show ChIP analyses of HIF-1α and rabbit IgG immunoprecipitates with PCR amplification of fragment containing *VEGF* HRE. Graphs show quantification of HIF-1α immunoprecipitates and data represent mean ± s.e.m. from at least three independent experiments.

HEXIM1 inhibits E_2-induced HIF-1α recruitment to the HRE in the VEGF *promoter*

Estrogens induce the recruitment of HIF-1α to the *VEGF* promoter in the rat uterus and in endometrial cancer cells (Kazi *et al*., 2005; Molitoris *et al*., 2009). To verify that E_2 induces HIF-1α recruitment to the *VEGF* promoter in the context of breast epithelial cells in our studies, we carried out ChIP assays using MCF-7 cells that were treated with vehicle or E_2 and grown under 21% O_2 or hypoxic (0.5% O_2) conditions. We found that E_2 enhanced the recruitment of HIF-1α to HRE in the *VEGF* promoter under 0.5% O_2 (Figure 3a). We also observed that some HIF-1α was immunoprecipitated under 21% O_2 conditions in MCF-7 cells (Figure 3a), suggesting that HIF-1α binds to the *VEGF* HRE under these conditions and may have a role in regulating VEGF expression, but comparatively, under 0.5% O_2 we observed a significantly enhanced amount of HIF-1α present at the *VEGF* HRE. In MCF-7 cells treated with hypoxia mimetic, cobaltous chloride ($CoCl_2$) (Cho *et al*., 2005), we observed a similar E_2-induced increase in HIF-1α recruitment to the *VEGF* HRE (Figure 3a).

To determine the effect of HEXIM1 on E_2-induced HIF-1α recruitment to the *VEGF* HRE in the presence or absence of ER-α, we carried out ChIP assays with MCF-7 and MDA-MB-231 cells. Under both 21 and 1% O_2, E_2 induced the recruitment of HIF-1α to the *VEGF* HRE in MCF-7 cells and increased HEXIM1 expression resulted in a decrease in E_2-induced recruitment of HIF-1α to the *VEGF* HRE (Figure 3b) likely due to a decrease in HIF-1α protein expression under hypoxia (Figure 2b). In MDA-MB-231 cells, hypoxia induced an increased HIF-1α recruitment to the *VEGF* HRE, but neither E_2 nor HEXIM1 significantly altered its occupancy on DNA (Figure 3c). Taken together, these data suggest that HEXIM1 regulates VEGF transcription through both ER-α- and HIF-1α-dependent mechanisms.

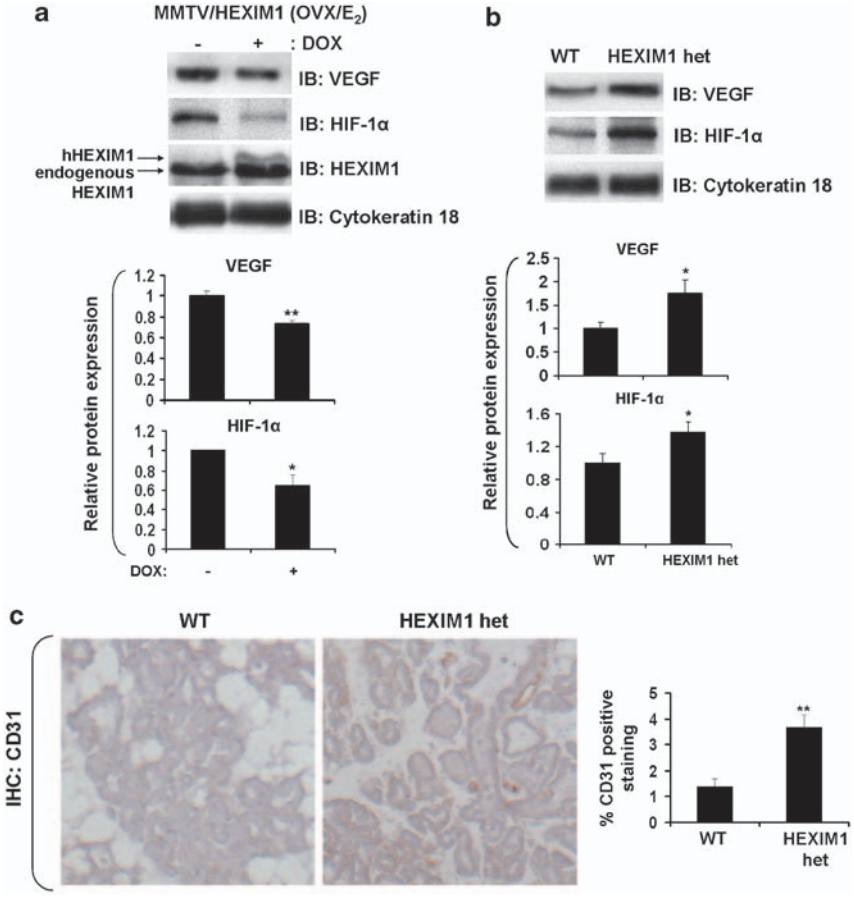

Figure 4 HEXIM1 modulates VEGF and HIF-1α expression and vascularization in mouse mammary gland. (**a**) MMTV/HEXIM1 mice were treated as described in the Materials and methods, and mammary gland tissue extracts were subjected to western blot. Antibodies for VEGF, HIF-1α and HEXIM1 were used for immunoblotting. Anti-cytokeratin 18 was used as an epithelial cell marker and a loading control. Graph panel shows quantification of VEGF and HIF-1α expression from mice treated with or without doxycycline (DOX). Data represent mean ± s.e.m. from four mice per group (± DOX) '*' and '**' indicate statistical significance ($P<0.05$ and $P<0.005$, respectively). (**b**) Western blot analyses of mammary gland extracts from adult wild-type mice (WT) and mice heterozygous for the HEXIM11-312 mutant allele (HEXIM1 het) using VEGF and HIF-1α antibodies. Blots were probed for cytokeratin 18 to normalize for epithelial cell content. Graph shows quantification of VEGF expression from WT and HEXIM11-312 heterozygous mice. Data represent mean ± s.e.m. from eight mice per group (VEGF) and at least three mice per group (HIF-1α); '*' indicates statistical significance ($P<0.05$). (**c**) Immunohistochemical detection of CD31 in mammary glands of lactating adult WT or HEXIM1 het mice. The panel is representative of four mice per group and graph shows quantification of %CD31-positive staining with mean ± s.e.m.; '**' indicates statistical significance ($P<0.005$).

HEXIM1 modulates VEGF and HIF-1α expression and angiogenesis in the mouse mammary gland independent of P-TEFb

To determine whether increased HEXIM1 expression significantly alters E_2-regulated VEGF and HIF-1α expression in the mammary gland, we extracted mammary glands from MMTV/HEXIM1 transgenic mice used in previous studies (Ogba *et al*., 2008). These mice inducibly overexpress HEXIM1 in the mammary gland when treated with doxycycline (+DOX) and were ovariectomized and treated with E_2 to monitor changes in gene expression that are modulated by E_2 (Ogba *et al*., 2008). We found that increased HEXIM1 expression (+DOX) significantly decreased VEGF and HIF-1α protein expression in the mammary gland (Figure 4a).

To verify the physiological relevance of HEXIM1 regulation on VEGF in the mammary gland, we generated mice expressing a knock-in mutation of HEXIM1 that have been previously described (Montano *et al*., 2008). HEXIM1 is expressed at full length as a 359-amino acid protein, with the C-terminus containing inhibitory domains for ER-α and P-TEFb (Wittmann *et al*., 2005; Zhou and Yik, 2006). These mice carry an insertional mutation in HEXIM1 that disrupts the C-terminus ($HEXIM1_{1-312}$). To demonstrate that the C-terminus mutation in HEXIM1 did not disrupt its potential to inhibit P-TEFb activity, we carried out *in vitro* kinase assays to compare the activity of wild-type HEXIM1 with $HEXIM1_{1-312}$ in Chinese hamster ovary cells. We found that $HEXIM1_{1-312}$ inhibits P-TEFb activity comparable to wild-type HEXIM1 (Supplementary Figure 4). Previous studies showed that $HEXIM1_{1-312}$ interacts with P-TEFb (Montano *et al*., 2008). *In vitro* studies verified this and we also found that $HEXIM1_{1-312}$ interacts with

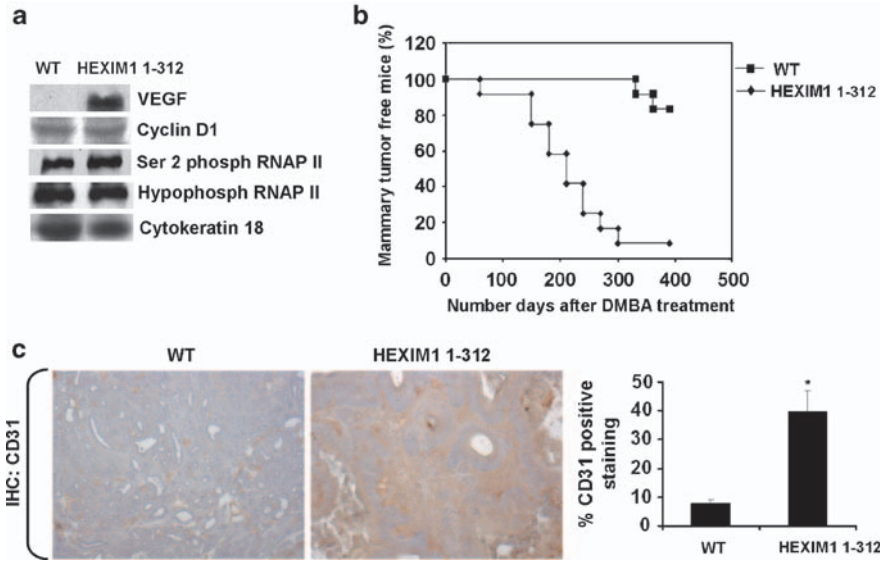

Figure 5 Expression of HEXIM1 C-terminus mutant enhances carcinogen-induced mammary tumorigenesis and correlates with increased vascularization of tumors. (**a**) Mammary gland extracts from adult WT mice and mice homozygous for the HEXIM11-312 mutant allele (HEXIM11-312) were subjected to western blot analyses using antibodies for VEGF, cyclin D1, serine 2 phosphorylated and hypophosphorylated RNAP II. Blots were probed for cytokeratin 18 to normalize for epithelial cell content. The panel is representative of at least three mice per group. (**b**) The graph describes DMBA-induced tumor incidence in HEXIM11-312 mice and their WT littermates assessed by palpitation and histopathological examination of excised tumors. DMBA was administered at 8 weeks of age by oral gavage. The frequency of palpable mammary tumors in HEXIM11-312 mice was statistically significant from that of the WT mice ($P<0.001$); ($n=12$ mice per group). (**c**) Immunohistochemical detection of CD31 in DMBA-induced mammary tumors excised from adult WT or HEXIM11-312 mice. The panel is representative of at least three mice per group and graph shows quantification of percent CD31-positive staining with mean ± s.e.m.; '*' indicates statistical significance ($P<0.05$).

ER-α (Supplementary Figure 5a, lanes 10–12). However, $HEXIM1_{1-312}$ has a diminished capacity to inhibit ER-α transcriptional activity (Supplementary Figure 5b). In the mammary glands of mice carrying the heterozygous allele for $HEXIM1_{1-312}$ (HEXIM1 het), we observed increases in VEGF and HIF-1α protein expression when compared with their wild-type littermates (Figure 4b). Taken together, these data suggest that the effects of the HEXIM1 insertional mutation on VEGF and HIF-1α protein expression are not due to a dysregulation of its P-TEFb-inhibitory function and that HEXIM1 modulates VEGF and HIF-1α expression in the mammary gland.

As VEGF is a proangiogenic factor, we wanted to determine whether the enhanced expression of VEGF in the mammary glands of HEXIM1 het mice corresponded to increased vascularization in the mammary gland. A hallmark of angiogenesis is the presence of platelet endothelial cell adhesion molecule-1 or cluster of differentiation molecule 31 (CD31) on the cell surface of endothelial cells (Woodfin *et al*., 2007). To carry out this, we examined any changes in CD31 expression in mammary glands from lactating HEXIM1 het mice and their wild-type lactating littermates using immunohistochemistry. We used mammary glands from lactating mice because increased vascularization and VEGF expression is critical for alveolar development and milk production (Rossiter *et al*., 2007). We found that there was an increase in CD31-positive staining in the mammary glands of HEXIM1 het mice when compared with their wild-type littermates (Figure 4c).

In addition, in the mammary glands of mice homozygous for the HEXIM1 mutant allele ($HEXIM1_{1-312}$), we observed increased VEGF protein expression when compared with their wild-type littermates (Figure 5a). There were no corresponding changes in cyclin D1 and the serine 2 phosphorylated form of RNAP II (Ser2 phosph RNAP II) protein levels (Figure 5a), which have been used as markers of P-TEFb activity *in vivo* (Sims *et al*., 2004; Ogba *et al*., 2008). To characterize the effect of enhanced VEGF expression in the mammary gland, we looked for any differences in epithelial cell proliferation in the mammary glands of $HEXIM1_{1-312}$ mice and their wild-type littermates and found that there was no significant difference in epithelial cell proliferation, as detected by bromodeoxyuridine incorporation (Supplementary Figure 6). Taken together, the data indicate that HEXIM1 regulates VEGF and HIF-1α expression in mammary cells and this in turn regulates the development of blood vessels in the mammary gland. In addition, the regulation of VEGF by HEXIM1 in breast cells seems to have a P-TEFb-independent component.

HEXIM1 C-terminus mutation increases incidence of carcinogen-induced mammary tumorigenesis and correlates with increased tumor vascularization

During tumorigenesis, hypoxic environments within the tumor enhance VEGF secretion and facilitate migration and proliferation of endothelial cells at the tumor site (Ferrara *et al*., 2003; Kimbro and Simons, 2006). To

determine the effect of the HEXIM1 C-terminus mutant on tumorigenesis, we used a well-known experimental model of carcinogen-induced mammary tumors (Li *et al*., 1995) and treated $HEXIM1_{1-312}$ mice and their wild-type littermates with sub-threshold levels of the carcinogen 7,12-dimethylbenz(a)anthracene through oral gavage. Moreover, the $HEXIM1_{1-312}$ mice are in the C57/BL6 background strain that is known to be relatively resistant to carcinogen-induced tumors (Lydon *et al*., 1999). We found that the $HEXIM1_{1-312}$ mice developed mammary tumors at a significantly higher incidence ($P<0.001$) than their wild-type littermates (Figure 5b).

To determine whether enhanced vascularization of the mammary tumors may contribute to or is associated with the increase in tumor incidence in $HEXIM1_{1-312}$ mice, mammary tumors that developed were excised and processed for immunohistochemistry to detect any changes in vascularization. We found that tumors from $HEXIM1_{1-312}$ mice exhibited increased vascularization, as evidenced by an increase in CD31-positive staining, when compared with their wild-type littermates (Figure 5c). Taken together, the *in vivo* data show that the HEXIM1 C-terminus mutation enhances mammary tumorigenesis and associated angiogenesis.

Discussion

This study demonstrates that HEXIM1 regulates VEGF expression in breast cancer cells through transcriptional regulation of ER-α and a regulation of HIF-1α expression under hypoxic conditions. We also demonstrate that HEXIM1 regulation of VEGF and HIF-1α occurs in the mouse mammary gland and leads to a modulation of mammary gland and tumor-associated angiogenesis. Taken together, these data support a novel role for HEXIM1 in mammary gland development and in mammary tumorigenesis partly through its effect on VEGF and angiogenesis.

Studies support the regulation of VEGF expression and angiogenesis in the female reproductive system and in breast cancer by estrogens, but specific mechanisms of the regulation are not always clear and E_2 has been shown to have variable effects on VEGF expression in breast cancer cells (Bogin and Degani, 2002; Garvin *et al*., 2005; Hyder, 2006). Nonetheless, estrogen-responsive elements have been identified in the *VEGF* gene, and the recruitment of E_2/ER-α to these regions has also been reported in breast, uterine and endometrial cancer cells (Stoner *et al*., 2004; Kazi *et al*., 2005; Molitoris *et al*., 2009). It has also been reported that direct interaction between the tumor suppressor, BRCA1, and ER-α inhibits E_2-driven VEGF transcription and secretion in breast cancer cells (Kawai *et al*., 2002). In other studies, we have shown that HEXIM1 is an ER-α-interacting protein and tumor suppressor (Wittmann *et al*., 2003, 2005). In this study, we found that increased HEXIM1 expression inhibited E_2-induced VEGF transcription through inhibition of ER-α recruitment to the *VEGF* promoter in breast cancer cells. HEXIM1 also interacts with and inhibits the activity of the P-TEFb to regulate gene expression (Wittmann *et al*., 2005; Zhou and Yik, 2006; Ogba *et al*., 2008). Other studies also support the P-TEFb-independent functions of HEXIM1 (Shimizu *et al*., 2005; Montano *et al*., 2008). In this study, we found that both E_2 and HEXIM1 did not affect P-TEFb recruitment to the GC-rich/Sp1 region of the *VEGF* promoter. The data do not rule of a functional role for P-TEFb in E_2/ER-α-regulated VEGF transcription, as our ChIP analyses was limited to examining the recruitment of P-TEFb to a specific E_2-responsive region of the *VEGF* promoter. However, it does suggest that P-TEFb may not be involved in the regulation of VEGF by HEXIM1 at GC-rich/Sp1 region in the *VEGF* promoter.

Hypoxia, a strong inducer of VEGF expression, regulates VEGF transcription through HIF-1α binding to HRE in the *VEGF* promoter (Bos *et al*., 2004; Kimbro and Simons, 2006). HIF-1α also has a role in tumor progression and metastasis (Liao *et al*., 2007). As both estrogen and hypoxia are involved in tumor development and progression, it is thought that they enhance VEGF expression in concert (Maity *et al*., 2001; Seifeddine *et al*., 2007). In this study, we found that HEXIM1 also inhibits VEGF transcription under hypoxic conditions through a decrease in E_2-induced HIF-1α protein expression and a decrease in E_2-induced HIF-1α recruitment to the HRE in the *VEGF* promoter. Previous studies have shown that E_2-induced VEGF transcription in uterine cells is initiated rapidly through the phosphatidylinositol 3-kinase/Akt pathway with a concurrent integration of ER-α and HIF-1α signaling (Kazi *et al*., 2005, 2009; Kazi and Koos, 2007). E_2 also induces increases in HIF-1α mRNA and protein expression in the uterus, which contributes to an increase in VEGF expression (Kazi *et al*., 2005, 2009). However, in this study, E_2 induces increases in HIF-1α protein expression in ER-α expressing breast cancer cells without significantly affecting HIF-1α mRNA levels. In addition, increased HEXIM1 expression decreased E_2-induced HIF-1α protein expression, without any effect on HIF-1α mRNA levels. Interestingly, the inhibition of E_2-induced HIF-1α protein occurred only under low oxygen conditions. This suggests that HEXIM1 modulates E_2-induced HIF-1α protein stability under hypoxia and warrants further study.

In addition, we demonstrate a physiological relevance for HEXIM1 regulation of VEGF expression *in vivo*. Increased HEXIM1 expression in the mammary gland of MMTV/HEXIM1 transgenic mice leads to a decrease in estrogen-driven VEGF and HIF-1α protein expression. Conversely, mutation of the C-terminus of HEXIM1 ($HEXIM1_{1-312}$) in mice led to enhanced VEGF and HIF-1α protein expression and vascularization in the mammary gland. In previous studies, we found that a deletion mutant form of HEXIM1 ($HEXIM_{1-310}$) was unable to inhibit E_2/ER-α transcriptional activity (Wittmann *et al*., 2005). In this study, we found that $HEXIM_{1-312}$ also has decreased the capacity to inhibit E_2/ER-α transcriptional activity. This suggests

that the regulation of VEGF expression by HEXIM1 in the mouse mammary gland could be due to a dysregulation of ER-α, which would be consistent with our breast epithelial cell studies, and indicate that HEXIM1 inhibits the actions of ER-α and HIF-1α, two positive regulators of VEGF gene transcription. We deduced that this regulation is independent of P-TEFb, given that $HEXIM1_{1-312}$ was found to inhibit P-TEFb activity but did not seem to have an effect on the expression levels of cyclin D1 and serine 2 phosphorylated RNAP II in the mammary gland, which have been shown to be reflective of changes in P-TEFb activity in previous studies (Ogba *et al*., 2008). However, we cannot completely rule out a role for P-TEFb in the regulation of VEGF transcription, as many genes contribute to VEGF transcriptional activity but are beyond the scope of our current study.

VEGF also has an important role in endothelial cell migration and proliferation and the resulting angiogenesis contributes to physiological and pathological processes (Ellis and Hicklin, 2008). We reported that a HEXIM1 C-terminus mutation leads to a decrease in VEGF expression in the developing mouse heart, which occurs through the attenuation of the inhibitory effect of C/EBPα by HEXIM1 on VEGF gene transcription in cardiomyocytes (Montano *et al*., 2008). In this study, we found that the $HEXIM1_{1-312}$ mice have increased susceptibility to developing carcinogen-induced mammary tumors that exhibit increased vascularization when compared with their wild-type littermates. The fact that HEXIM1 displays different effects on VEGF in cardiomyocytes compared with mammary epithelial cells suggests that there may be tissue-specific factors that associate with HEXIM1 to regulate VEGF expression. Nonetheless, taken together, our findings support a novel role for HEXIM1 during mammary gland development and tumorigenesis as illustrated in our model (Figure 6).

VEGF-targeted therapy includes targeting circulating VEGF and VEGF receptor blockade (Ellis and Hicklin, 2008). As cancer cells typically develop resistance to cancer therapeutic drugs, it is important to identify potential targets with multiple mechanisms of action. In breast cancer cells, HEXIM1 regulates proliferation (Wittmann *et al*., 2005; Ogba *et al*., 2008) and, as we show in this study, angiogenesis—two critical processes that occur during mammary tumorigenesis. Specifically, we found that HEXIM1 modulates E_2-driven VEGF expression in breast cells through transcriptional regulation of ER-α and regulation of HIF-1α protein expression, with functional consequences on angiogenesis in the mammary gland and during carcinogen-induced mammary tumorigenesis. These studies elucidate the reasons why HEXIM1 might be a desirable therapeutic target for breast cancer. Future studies will aim to investigate the specific mechanisms of HEXIM1 regulation of HIF-1α expression and how it contributes to tumor progression.

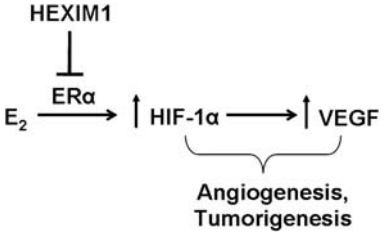

Figure 6 Model: HEXIM1 regulates VEGF expression through ER-α and HIF-1α to modulate angiogenesis and tumorigenesis.

Materials and methods

Reverse transcriptase–PCR analyses
MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained as previously described (Wittmann *et al*., 2003, 2005). MCF-7 and MDA-MB-231 cells were transiently transfected with pCMV-Tag2B, pCMV-Tag2B-ER-α or pCMV-Tag2B-HEXIM1 using FuGENE HD transfection reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Cells were treated with E_2 and grown under high (21%) or low (1%) oxygen conditions as indicated. All cells were subsequently subjected to reverse transcriptase PCR analyses as previously described (Ogba *et al*., 2008). PCR products were run in 2.5% agarose gels and visualized by ethidium bromide staining. A 12-bit digital camera captured fluorescence and signal intensities were quantified using the Alphaimager software from Alpha Innotech (San Leandro, CA, USA). Signals from genes of interest were normalized to signals from GAPDH and presented as 'relative mRNA expression.' The primers used and sequences are described in the Supplementary data.

Western analyses
MCF-7 and MDA-MB-231 cells were treated as described and total protein was extracted from cells and used for western blot analysis as previously described (Ogba *et al*., 2008).

ChIP assays
MCF-7 and MDA-MB-231 cells were transfected as described with pCMV-Tag2B, pCMV-Tag2B-ER-α or pCMV-Tag2B-HEXIM1 using FuGENE HD transfection reagent (Roche). Cells were then treated with ethanol or 100 nM E_2 and grown under high (21%) or low (1%) oxygen conditions as indicated. ChIP assays were carried out as previously described (Ogba *et al*., 2008). Signals from specific immunoprecipitations were normalized to signals from input DNA and presented as 'fold enrichment' relative to signals from untreated and untransfected sample groups set at '1'. The primers used and sequences are described in the Supplementary data.

CTD kinase assays
Kinase assays were carried out as previously described (Ogba *et al*., 2008). See Supplementary data for complete description of assay.

Mice studies
MMTV/HEXIM1 transgenic mice were generated and treated as previously described (Ogba *et al*., 2008). $HEXIM1_{1-312}$ knock-in mutant mice were generated as previously described (Montano *et al*., 2008). Details of the studies carried out examining the effect of the HEXIM1 mutation on vascularization

of the developing mammary gland and mammary tumors are described in the Supplementary data.

Immunohistochemistry
Immunohistochemistry using sections from mammary glands and tumors are described in the Supplementary data.

Data analyses
Data points in figures represent the means ± s.e.m. based on at least three independent experiments carried out in duplicate. The statistical significance was determined using Student's *t* test comparison for unpaired data and was indicated as follows: $*P<0.05$; $**P<0.005$; $***P<0.0005$; $****P<0.00005$.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)