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

Ndiya Ogba¹, Yong Qiu Doughman³, Laura J. Chaplin¹, Yanduan Hu¹, Madhusudhana Gargesha², Michiko Watanabe³, and Monica M. Montano¹

¹Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106
²Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106
³Department of Pediatrics, Rainbow Babies and Children’s Hospital, Cleveland, OH 44106

Abstract

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 due to 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 via inhibition of Estrogen Receptor alpha 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°°°°°°°°°) led to increased VEGF and HIF-1α expression and vascularization in mammary glands of heterozygous HEXIM1°°°°°°°°° mice when compared to their wild-type litters. Additionally, HEXIM1°°°°°°°°° 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.

Keywords

HEXIM1; VEGF; HIF-1α; P-TEFb; angiogenesis; mammary tumors

Conflict of Interest

The authors declare no conflict of interest.
Introduction

Estrogens play a significant role in the etiology and progression of breast cancers and mediate their actions through estrogen receptors (ERs), ERα and ERβ, nuclear steroid receptors that regulate transcription either directly by binding to estrogen-response elements of target genes or indirectly via protein-protein interactions with other transcription factors (Deroo and Korach, 2006; Kushner et al., 2000). Another factor known to play 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 (Ellis and Hicklin, 2008; Rugo, 2004).

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 ERα-regulatory components (Kazi et al., 2005; Stoner et al., 2004). In addition, many tumors co-express ERα 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 due to 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 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 ERα-positive and negative breast cancers (Bos et al., 2004; Kimbro and Simons, 2006; Liao et al., 2007). Also, 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 ERα 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) via cyclin T1 and inhibits its activity (Zhou and Yik, 2006). P-TEFb is a complex of cyclin T1 and cyclin-dependent kinase 9 (CDK9) 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 ERα target genes (Ogba et al., 2008). HEXIM1 regulation of these genes involves inhibiting P-TEFb activity and recruitment to the ERα target genes (Ogba et al., 2008). In addition, recent work from our laboratory uncovered a novel role for 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 via ERα in breast cancer cells, it is not known whether this regulation is dependent on P-TEFb. Also, 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 via its effect on transcription factors ERα 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 ERα-positive, were transfected with empty vector or pCMV-Tag2B-HEXIM1 and treated with ethanol (vehicle) or increasing concentrations of 17-beta estradiol (E2). There was a significant increase in VEGF mRNA expression in cells treated with 1 and 10 nM E2 (Figure 1A, lanes 2 and 3). Although the E2-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 E2-induced increases in VEGF mRNA within a similar range (Higgins et al., 2006; Maity et al., 2001). The MCF-7 cells were adequately responsive to E2 as there were significant increases in other ER target genes, including Trefoil factor 1 (TFF1 or pS2) and progesterone receptor (PR) (Supplemental Figure 1). Increased HEXIM1 expression in MCF-7 cells inhibited E2-induced increases in VEGF mRNA expression (Figure 1A, lanes 5 and 6). Increased HEXIM1 expression also inhibited E2-induced pS2 mRNA expression but did not affect E2-induced PR mRNA expression as previously reported (Supplemental Figure 1) (Ogba et al., 2008).

From previous studies, we know that HEXIM1 inhibits E2/ERα transcriptional activity in the context of some ERα target genes (Ogba et al., 2008; Wittmann et al., 2005). In ERα-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 E2/ERα. To further test this hypothesis, we transfected MDA-MB-231 cells with control vector or pCMV-Tag2B-ERα and treated cells with vehicle or E2. MDA-MB-231 cells are typically not E2-responsive except in some cases of ERα or ERβ-overexpression (Buteau-Lozano et al., 2002), but in our hands, we found that VEGF mRNA was slightly, but significantly responsive to E2 in ERα-expressing MDA-MB-231 cells (Supplemental Figure 2, lane 3) and increased HEXIM1 expression inhibited the E2-induced increase in VEGF mRNA expression in these cells (Supplemental 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 E2-induced secretion of VEGF protein from MCF-7 cells (Figure 1C). Taken together, these data suggest that HEXIM1 regulation of VEGF expression requires E2/ERα.
Increased HEXIM1 expression inhibits the recruitment of E₂/ERα 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 E₂/ERα-driven VEGF transactivation (Kazi et al., 2005; Stoner et al., 2004). To determine the effect of HEXIM1 on E₂/ERα regulated VEGF transcription, we carried out chromatin immunoprecipitation (ChIP) assays to examine changes in the recruitment of ERα 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 appear to be E₂-dependent (Figure 1E). This increased occupancy of HEXIM1 led to a decrease in the recruitment of E₂/ERα and RNA polymerase II to the VEGF promoter (Figure 1E and Supplemental Figure 3A). As a control, we examined the recruitment of ERα 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 (Supplemental Figure 3B).

Previous studies from our laboratory have determined that HEXIM1 inhibits E₂-driven transcription of some ERα target genes in a P-TEFb-dependent manner (Ogba et al., 2008; Wittmann et al., 2005). To determine whether P-TEFb is involved in E₂/ERα-driven VEGF transcription, we immunoprecipitated cyclin T1 in the ChIP assays and found that cyclin T1 recruitment was not enhanced by E₂ treatment and increased HEXIM1 expression did not significantly affect its occupancy at the GC-rich/Sp1 region in the VEGF promoter (Figure 1E and Supplemental Figure 3A). We also examined the recruitment of cyclin T1 to the VEGF promoter control region described earlier and found that cyclin T1 was also not recruited to this region (Supplemental Figure 3B). Although it is possible that E₂ 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 E₂ and not significantly affected by HEXIM1.

Under hypoxia, HEXIM1 inhibition of VEGF expression correlates with a decrease in E₂-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 E₂, we transfected MCF-7 cells with control vector or Flag-HEXIM1 expression vector and subjected the cells to either high oxygen (21% O₂) or low oxygen (1% O₂) conditions. We found that increased HEXIM1 expression inhibited E₂-induced increases in VEGF mRNA expression under both 21% and 1% O₂ conditions (Figure 2A). However, under 1% O₂ 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 E₂ 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 E₂ and hypoxia (1% O₂) induced a slightly higher fold increase in VEGF mRNA expression when compared to E₂ under 21% O₂ conditions that is statistically significant (p < 0.05) (Figure 2A). However, there was no change in HIF-1α mRNA expression in response to E₂ treatment or increased HEXIM1 expression under 21% and 1% O₂ conditions (Figure 2A) suggesting that the
effect of HEXIM1 on HIF-1α is probably via post-translational regulation of HIF-1α. Western blot analyses showed E2 induced an increase in HIF-1α protein expression (Figure 2B). However, increased HEXIM1 expression inhibited E2-induced increases in HIF-1α protein expression under hypoxia (Figure 2B, compare lanes 6 and 8). In MDA-MB-231 cells, we found that E2 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 E2/ERα regulates HIF-1α protein expression to enhance VEGF expression in breast cancer cells and that HEXIM1 modulates E2/ERα-regulated HIF-1α protein expression under low oxygen levels.

**HEXIM1 inhibits E2-induced HIF-1α recruitment to the hypoxia-response element 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 E2 induces HIF-1α recruitment to the VEGF promoter in the context of breast epithelial cells in our studies, we performed ChIP assays using MCF-7 cells that were treated with vehicle or E2 and grown under 21% O2 or hypoxic (0.5% O2) conditions. We found that E2 enhanced the recruitment of HIF-1α to the hypoxia response element (HRE) in the VEGF promoter under 0.5% O2 (Figure 3A). We also observed that some HIF-1α was immunoprecipitated under 21% O2 conditions in MCF-7 cells (Figure 3A), suggesting that HIF-1α binds to the VEGF HRE under these conditions and may play a role in regulating VEGF expression, but comparatively, under 0.5% O2 we observed a significantly enhanced amount of HIF-1α present at the VEGF HRE. In MCF-7 cells treated with hypoxia mimetic, cobaltous chloride (CoCl2) (Cho et al., 2005), we observed a similar E2-induced increase in HIF-1α recruitment to the VEGF HRE (Figure 3A).

To determine the effect of HEXIM1 on E2-induced HIF-1α recruitment to the VEGF HRE in the presence or absence of ERα, we carried out ChIP assays with MCF-7 and MDA-MB-231 cells. Under both 21% and 1% O2, E2 induced the recruitment of HIF-1α to the VEGF HRE in MCF-7 cells and increased HEXIM1 expression resulted in a decrease in E2-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 increased HIF-1α recruitment to the VEGF HRE but neither E2 nor HEXIM1 significantly altered its occupancy on DNA (Figure 3C). Taken together, these data suggest that HEXIM1 regulates VEGF transcription via both ERα- and HIF-1α-dependent mechanisms.

**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 E2-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 E2 to monitor changes in gene expression that are modulated by E2 (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 ERα and P-TEFb (Wittmann et al., 2005; Zhou and Yik, 2006). These mice carry an insertional mutation in HEXIM1 that disrupts the C-terminus (HEXIM11-312). 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 to HEXIM11-312 in Chinese hamster ovary (CHO) cells. We found that HEXIM11-312 inhibits P-TEFb activity comparable to wild-type HEXIM1 (Supplemental Figure 4). Previous studies showed that HEXIM11-312 interacts with P-TEFb (Montano et al., 2008). In vitro studies verified this and we also found that HEXIM11-312 interacts with ERα (Supplemental Figure 5A, lanes 10-12). However, HEXIM11-312 has a diminished capacity to inhibit ERα transcriptional activity (Supplemental Figure 5B). In the mammary glands of mice carrying the heterozygous allele for HEXIM11-312 (HEXIM1 het), we observed increases in VEGF and HIF-1α protein expression when compared to 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.

Since VEGF is a proangiogenic factor, we wanted to determine if 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 (PECAM-1) or cluster of differentiation molecule 31 (CD31) on the cell surface of endothelial cells (Woodfin et al., 2007). To do 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 to their wild-type littermates (Figure 4C).

Additionally, in the mammary glands of mice homozygous for the HEXIM1 mutant allele (HEXIM11-312) we observed increased VEGF protein expression when compared to 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 (Ogba et al., 2008; Sims et al., 2004). 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 HEXIM11-312 mice and their wild-type littermates and found that there was no significant difference in epithelial cell proliferation, as detected by Bromodeoxyuridine (BrDU) incorporation (Supplemental 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. Additionally, the regulation of VEGF by HEXIM1 in breast cells appears 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 HEXIM11-312 mice and their wild-type littermates with sub-threshold levels of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) via oral gavage. Moreover, the HEXIM11-312 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 HEXIM11-312 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 HEXIM11-312 mice, mammary tumors that developed were excised and processed for immunohistochemistry to detect any changes in vascularization. We found that tumors from HEXIM11-312 mice exhibited increased vascularization, as evidenced by an increase in CD31 positive staining, when compared to 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$\alpha$ and a regulation of HIF-1$\alpha$ expression under hypoxic conditions. We also demonstrate that HEXIM1 regulation of VEGF and HIF-1$\alpha$ 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 $E_2/ER\alpha$ to these regions has also been reported in breast, uterine and endometrial cancer cells (Kazi et al., 2005; Molitoris et al., 2009; Stoner et al., 2004). It has also been reported that direct interaction between the tumor suppressor, BRCA1, and ER$\alpha$ 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$\alpha$-interacting protein and tumor suppressor (Wittmann et al., 2005; Wittmann et al., 2003). In this study, we found...
that increased HEXIM1 expression inhibited E2-induced VEGF transcription via inhibition of ERα 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 (Ogba et al., 2008; Wittmann et al., 2005; Zhou and Yik, 2006). Other studies also support P-TEFb independent functions of HEXIM1 (Montano et al., 2008; Shimizu et al., 2005). In this study, we found that both E2 and HEXIM1 did not affect P-TEFb recruitment to the GC-rich/Sp1 region of the VEGF promoter. The data does not rule out a functional role for P-TEFb in E2/ERα-regulated VEGF transcription, as our ChIP analyses was limited to examining the recruitment of P-TEFb to a specific E2-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 the hypoxia-inducible factor-1α (HIF-1α) binding the HRE in the VEGF promoter (Bos et al., 2004; Kimbro and Simons, 2006). HIF-1α also plays a role in tumor progression and metastasis (Liao et al., 2007). Since 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 via a decrease in E2-induced HIF-1α protein expression and a decrease in E2-induced HIF-1α recruitment to the HRE in the VEGF promoter. Previous studies have shown that E2-induced VEGF transcription in uterine cells is initiated rapidly through the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway with a concurrent integration of ERα and HIF-1α signaling (Kazi et al., 2005; Kazi and Koos, 2007; Kazi et al., 2009). E2 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; Kazi et al., 2009). However, in this study, E2 induces increases in HIF-1α protein expression in ERα expressing breast cancer cells without significantly affecting HIF-1α mRNA levels. Also, increased HEXIM1 expression decreased E2-induced HIF-1α protein expression, without any effect on HIF-1α mRNA levels. Interestingly, the inhibition of E2-induced HIF-1α protein occurred only under low oxygen conditions. This suggests that HEXIM1 modulates E2-induced HIF-1α protein stability under hypoxia and warrants further study.

Additionally, 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 (HEXIM11-312) 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 (HEXIM1-310) was unable to inhibit E2/ERα transcriptional activity (Wittmann et al., 2005). In this study, we found that HEXIM11-312 also has decreased capacity to inhibit E2/ERα transcriptional activity. This suggests that the regulation of VEGF expression by HEXIM1 in the mouse mammary gland could be due to a dysregulation of ERα, which would be consistent with our breast epithelial cell studies, and indicate that HEXIM1 inhibits the actions of ERα and HIF-1α, two positive regulators of VEGF gene transcription. We deduced that this
regulation is independent of P-TEFb, given that HEXIM1\textsubscript{1-312} was found to inhibit P-TEFb activity but did not appear 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 plays 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/EBP\alpha by HEXIM1 on VEGF gene transcription in cardiomyocytes (Montano et al., 2008). In our current study, we found that the HEXIM1\textsubscript{1-312} mice have increased susceptibility to developing carcinogen-induced mammary tumors that exhibit increased vascularization when compared to their wild-type littermates. The fact that HEXIM1 is displaying different effects on VEGF in cardiomyocytes versus the 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). Since 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 factors that contribute to proliferation (Ogba et al., 2008; Wittmann et al., 2005) and as we show in this study angiogenesis—two critical processes that occur during mammary tumorigenesis. Specifically, we found that HEXIM1 modulates E\textsubscript{2}-driven VEGF expression in breast cells through transcriptional regulation of ER\alpha and regulation of HIF-1\alpha protein expression, with functional consequences on angiogenesis in the mammary gland and during carcinogen-induced mammary tumorigenesis. These studies elucidate 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-1\alpha expression and how it contributes to tumor progression.

Materials and Methods

Reverse Transcription (RT) PCR Analyses

MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained as previously described (Wittmann et al., 2005; Wittmann et al., 2003). MCF-7 and MDA-MB-231 cells were transiently transfected with pCMV-Tag2B, pCMV-Tag2B-ER\alpha, or pCMV-Tag2B-HEXIM1 using FuGENE HD transfection reagent (Roche, IN) according to the manufacturer's instructions. Cells were treated with 17-beta estradiol (E\textsubscript{2}) and grown under high (21%) or low (1%) oxygen conditions as indicated. All cells were subsequently subjected to reverse transcription-PCR (RT-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). 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).

Chromatin immunoprecipitation (ChIP) assays

MCF-7 and MDA-MB-231 cells were transfected as described with pCMV-Tag2B, pCMV-Tag2B-ERα, or pCMV-Tag2B-HEXIM1 using FuGENE HD transfection reagent (Roche, IN). Cells were then treated with ethanol or 100 nM E2 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 performed examining the effect of the HEXIM1 mutation on vascularization of the developing mammary gland and mammary tumors are described in Supplementary data.

Immunohistochemistry

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

Data analyses

Data points in figures represent the means ± s.e.m. (standard error mean) based on at least three independent experiments performed 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.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Drs. Anthony J. Berdis and Jay Prendergast for reagents and their help with kinase and ELISA assays. This work was supported by National Institute of Health grant CA92440 and American Heart Association grant to M.M.M and a Department of Defense Predoctoral Fellowship W81XWH-06-1-0426 to N.O.

References

Bogin L, Degani H. Hormonal regulation of VEGF in orthotopic MCF7 human breast cancer. Cancer Res. 2002; 62:1948–51. [PubMed: 11929808]

Bos R, van Diest PJ, van der Groep P, Shvarts A, Greijer AE, van der Wall E. Expression of hypoxia-inducible factor-1alpha and cell cycle proteins in invasive breast cancer are estrogen receptor related. Breast Cancer Res. 2004; 6:R450–9. [PubMed: 15217513]

Buteau-Lozano H, Ancelin M, Lardeux B, Milanini J, Perrot-Apllanat M. Transcriptional regulation of vascular endothelial growth factor by estradiol and tamoxifen in breast cancer cells: a complex interplay between estrogen receptors alpha and beta. Cancer Res. 2002; 62:4977–84. [PubMed: 12208749]

Cho J, Kim D, Lee S, Lee Y. Cobalt chloride-induced estrogen receptor alpha down-regulation involves hypoxia-inducible factor-1alpha in MCF-7 human breast cancer cells. Mol Endocrinol. 2005; 19:1191–9. [PubMed: 15695373]

Deroo BJ, Korach KS. Estrogen receptors and human disease. J Clin Invest. 2006; 116:561–70. [PubMed: 16511588]

Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer. 2008; 8:579–91. [PubMed: 18596824]

Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003; 9:669–76. [PubMed: 12778165]

Garvin S, Nilsson UW, Dabrosin C. Effects of oestradiol and tamoxifen on VEGF, soluble VEGFR-1, and VEGFR-2 in breast cancer and endothelial cells. Br J Cancer. 2005; 93:1005–10. [PubMed: 16234819]

Higgins KJ, Liu S, Abdelrahim M, Yoon K, Vanderlaag K, Porter W, et al. Vascular endothelial growth factor receptor-2 expression is induced by 17beta-estradiol in ZR-75 breast cancer cells by estrogen receptor alpha/Sp proteins. Endocrinology. 2006; 147:3285–95. [PubMed: 16574784]

Hyder SM. Sex-steroid regulation of vascular endothelial growth factor in breast cancer. Endocr Relat Cancer. 2006; 13:667–87. [PubMed: 16954424]

Kawai H, Li H, Chun P, Avraham S, Avraham HK. Direct interaction between BRCA1 and the estrogen receptor regulates vascular endothelial growth factor (VEGF) transcription and secretion in breast cancer cells. Oncogene. 2002; 21:7730–9. [PubMed: 12400015]

Kazi AA, Jones JM, Koos RD. Chromatin immunoprecipitation analysis of gene expression in the rat uterus in vivo: estrogen-induced recruitment of both estrogen receptor alpha and hypoxia-inducible factor 1 to the vascular endothelial growth factor promoter. Mol Endocrinol. 2005; 19:2006–19. [PubMed: 15774498]

Kazi AA, Koos RD. Estrogen-induced activation of hypoxia-inducible factor-1alpha, vascular endothelial growth factor expression, and edema in the uterus are mediated by the phosphatidylinositol 3-kinase/Akt pathway. Endocrinology. 2007; 148:2363–74. [PubMed: 17272396]

Kazi AA, Molitoris KH, Koos RD. Estrogen rapidly activates the PI3K/AKT pathway and hypoxia-inducible factor 1 and induces vascular endothelial growth factor A expression in luminal epithelial cells of the rat uterus. Biol Reprod. 2009; 81:378–87. [PubMed: 19420388]

Kimbro KS, Simons JW. Hypoxia-inducible factor-1 in human breast and prostate cancer. Endocr Relat Cancer. 2006; 13:739–49. [PubMed: 16954428]

Oncogene. Author manuscript; available in PMC 2010 December 01.
Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, et al. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol. 2000; 74:311–7. [PubMed: 11162939]

Li B, Kittrell FS, Medina D, Rosen JM. Delay of dimethylbenz[a]anthracene-induced mammary tumorigenesis in transgenic mice by apoptosis induced by an unusual p53 protein. Mol Carcinog. 1995; 14:75–83. [PubMed: 7576102]

Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. Cancer Res. 2007; 67:563–72. [PubMed: 17234764]

Lydon JP, Ge G, Kittrell FS, Medina D, O'Malley BW. Murine mammary gland carcinogenesis is critically dependent on progesterone receptor function. Cancer Res. 1999; 59:4276–84. [PubMed: 10485472]

Maity A, Sall W, Koch CJ, Oprysko PR, Evans SM. Low pO2 and beta-estradiol induce VEGF in MCF-7 and MCF-7-5C cells: relationship to in vivo hypoxia. Breast Cancer Res Treat. 2001; 67:51–60. [PubMed: 11518466]

Molitoris KH, Kazi AA, Koos RD. Inhibition of oxygen-induced hypoxia-inducible factor-1alpha degradation unmasks estradiol induction of vascular endothelial growth factor expression in ECC-1 cancer cells in vitro. Endocrinology. 2009; 150:5405–14. [PubMed: 19819950]

Montano MM, Doughman YQ, Deng H, Chaplin L, Yang J, Wang N, et al. Mutation of the HEXIM1 gene results in defects during heart and vascular development partly through downregulation of vascular endothelial growth factor. Circ Res. 2008; 102:415–22. [PubMed: 18079413]

Ogba N, Chaplin LJ, Doughman YQ, Fujinaga K, Montano MM. HEXIM1 regulates 17beta-estradiol/estrogen receptor-alpha-mediated expression of cyclin D1 in mammary cells via modulation of P-TEFb. Cancer Res. 2008; 68:7015–24. [PubMed: 18757415]

Rossiter H, Barresi C, Ghannadan M, Gruber F, Mildner M, Fodinger D, et al. Inactivation of VEGF in mammary gland epithelium severely compromises mammary gland development and function. FASEB J. 2007; 21:3994–4004. [PubMed: 17625068]

Rugo HS. Bevacizumab in the treatment of breast cancer: rationale and current data. Oncologist. 2004; 9(Suppl 1):43–9. [PubMed: 15178815]

Seifeddine R, Dreiem A, Tomkiewicz C, Fulchignoni-Lataud MC, Brito I, Danan JL, et al. Hypoxia and estrogen co-operate to regulate gene expression in T-47D human breast cancer cells. J Steroid Biochem Mol Biol. 2007; 104:169–79. [PubMed: 17475478]

Shimizu N, Ouchida R, Yoshikawa N, Hisada T, Watanabe H, Okamoto K, et al. HEXIM1 forms a transcriptionally abortive complex with glucocorticoid receptor without involving 7SK RNA and positive transcription elongation factor b. Proc Natl Acad Sci U S A. 2005; 102:8555–60. [PubMed: 15941832]

Sims RJ 3rd, Belotserkovskaya R, Reinberg D. Elongation by RNA polymerase II: the short and long of it. Genes Dev. 2004; 18:2437–68. [PubMed: 15489290]

Stoner M, Wormke M, Saville B, Samudio I, Qin C, Abdelrahim M, et al. Estrogen regulation of vascular endothelial growth factor gene expression in ZR-75 breast cancer cells through interaction of estrogen receptor alpha and SP proteins. Oncogene. 2004; 23:1052–63. [PubMed: 14647449]

Wittmann BM, Fujinaga K, Deng H, Ogba N, Montano MM. The breast cell growth inhibitor, estrogen down regulated gene 1, modulates a novel functional interaction between estrogen receptor alpha and transcriptional elongation factor cyclin T1. Oncogene. 2005; 24:5576–88. [PubMed: 15940264]

Wittmann BM, Wang N, Montano MM. Identification of a novel inhibitor of breast cell growth that is down-regulated by estrogens and decreased in breast tumors. Cancer Res. 2003; 63:5151–8. [PubMed: 12941847]

Woodfin A, Voisin MB, Nourshargh S. PECAM-1: a multi-functional molecule in inflammation and vascular biology. Arterioscler Thromb Vasc Biol. 2007; 27:2514–23. [PubMed: 17872453]

Zhou Q, Yik JH. The Yin and Yang of P-TEFb regulation: implications for human immunodeficiency virus gene expression and global control of cell growth and differentiation. Microbiol Mol Biol Rev. 2006; 70:646–59. [PubMed: 16959964]
Figure 1. Increased HEXIM1 expression inhibits E2-induced transcription of VEGF via ERα 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-beta estradiol (E2) for 4 hours. Graph shows fold change of VEGF mRNA expression levels measured by reverse transcriptase PCR (RT-PCR). Data represents mean±s.e.m. from 4 independent experiments performed 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 17-beta estradiol (E2) for 4 hours. Graph shows fold change of VEGF mRNA expression levels measured by reverse transcriptase PCR (RT-PCR). Data represents mean±s.e.m. from 3 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 E2 for 12 hours. Secreted VEGF protein levels were measured by ELISA. Data represents mean±s.e.m. from 4 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 E2 for 45 minutes. Results show ChIP analyses of lysates immunoprecipitated with ERα, 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 performed and graph shows quantification of PCR products as indicated. Data represents mean±s.e.m. from 3 independent experiments.
Figure 2. Increased HEXIM1 expression inhibits E2-induced VEGF mRNA expression under hypoxia that correlates with a decrease in E2-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 E2 and grown under high oxygen (21% O2) or low oxygen (1% O2) conditions for 12 hours. Results show fold change of VEGF mRNA expression levels measured by RT-PCR. Data represents mean±s.e.m. from 3 independent experiments performed 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 E2 and grown under 21% or 1% O2 conditions as indicated for at least 12 hours. Western blot analyses show changes in HIF-1α protein expression and protein expression of HEXIM1 and GAPDH (loading control). Data represents mean±s.e.m. from 4 independent experiments performed in duplicate; ‘**’ and ‘***’ represents 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 E2 and grown under 21% or 1% O2 conditions as indicated for at least 12 hours. Western blot analyses show changes in HIF-1α protein expression and protein expression of HEXIM1 and GAPDH (loading control). Data represents mean±s.e.m. from 3 independent experiments performed in duplicate.
Figure 3. Increased HEXIM1 expression inhibits E2-induced recruitment of HIF-1α to VEGF Hypoxia Response Element

A. MCF-7 cells were treated with ethanol or 100 nM E2 and subjected to high (21%) or low (0.5%) oxygen conditions as indicated or treated with 100 μM cobaltous chloride (CoCl2) for 6 hours. 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 the hypoxic response element (HRE) in the VEGF promoter (region indicated in Figure 1D). Data represents mean±s.e.m. from 3 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 E2 and subjected to 21% or 1% O2 conditions as indicated for 16 hours. 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 represents mean±s.e.m. from at least 4 independent experiments; ‘*’ and ‘**’ indicates 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 E2 and subjected to 21% or 1% O2 conditions as indicated for 16 hours. 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 represents mean±s.e.m. from at least 3 independent experiments.

Ogba et al. Page 16

Oncogene. Author manuscript; available in PMC 2010 December 01.
Figure 4. HEXIM1 modulates VEGF and HIF-1α expression and vascularization in mouse mammary gland

A. MMTV/HEXIM1 mice were treated as described in 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 represents mean±s.e.m. from 4 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 HEXIM1 1-312 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 HEXIM1 1-312 heterozygous mice. Data represents mean±s.e.m. from 8 mice per group (VEGF) and at least 3 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. Panel is representative of 4 mice per group and graph shows quantification of %CD31-positive staining with mean±s.e.m.; ‘**’ indicates statistical significance (p < 0.005).
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 HEXIM1 1-312 mutant allele (HEXIM1 1-312) 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. Panel is representative of at least 3 mice per group.

B. The graph describes DMBA-induced tumor incidence in HEXIM1 1-312 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 HEXIM1 1-312 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 HEXIM1 1-312 mice. Panel is representative of at least 3 mice per group and graph shows quantification of %CD31-positive staining with mean±s.e.m.; ** indicates statistical significance ($p < 0.05$).
Figure 6.
Model: HEXIM1 regulates VEGF expression via ERα and HIF-1α to modulate angiogenesis and tumorigenesis.