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Inhibition of metastasis by HEXIM1 through effects on cell invasion and angiogenesis

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Abstract

We report on the role of Hexamethylene-bis-acetamide-inducible protein 1 (HEXIM1) as an inhibitor of metastasis. HEXIM1 expression is decreased in human metastatic breast cancers when compared to matched primary breast tumors. Similarly we observed decreased expression of HEXIM1 in lung metastasis when compared to primary mammary tumors in a mouse model of metastatic breast cancer, the Polyoma Middle-T antigen (PyMT) transgenic mouse. Re-expression of HEXIM1 (through transgene expression or localized delivery of a small molecule inducer of HEXIM1 expression, Hexamethylene-bis-acetamide) in PyMT mice resulted in inhibition of metastasis to the lung. Our present studies indicate that HEXIM1 downregulation of HIF-1α protein allows not only for inhibition of VEGF-regulated angiogenesis, but also inhibition of compensatory pro-angiogenic pathways and recruitment of bone marrow derived cells (BMDCs). Another novel finding is that HEXIM1 inhibits cell migration and invasion, that can be partly attributed to decreased membrane localization of the 67kDa laminin receptor, 67LR, and inhibition of the functional interaction of 67LR with laminin. Thus HEXIM1 re-expression in breast cancer has therapeutic advantages by simultaneously targeting more than one pathway involved in angiogenesis and metastasis. Our results also support the potential for HEXIM1 to indirectly act on multiple cell types to suppress metastatic cancer.

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Keywords

HEXIM1; breast cancer; metastasis; angiogenesis

INTRODUCTION

Angiogenesis-mediated progression form micrometastasis to macrometastasis is a major cause of death in cancer patients (1). Activation of the angiogenic switch involves increased expression and/or export of proangiogenic factors and/or down-regulation of antiangiogenic mediators (2). While VEGF is a major regulator of tumor angiogenesis, the clinical benefits of anti-VEGF therapy are relatively modest and usually measured in weeks or months (3). In some cases, patients do not respond to anti-VEGF treatments (4). Independence from the VEGF pathway is associated with hypoxia-mediated induction of proangiogenic factors such as FGF1 and 2, ephrin A1 and 2, and angiopoietin 1 (5, 6). HIF-1α is a major regulator of these angiogenic actors following hypoxia (7). Moreover, HIF-1α plays a role in the recruitment of bone marrow derived cells (BMDCs) that are sources of proangiogenic factors and support the growth of existing blood vessels (8).

We have previously reported that the transcription factor Hexamethylene-bis-acetamide-inducible (HMBA) protein 1 (HEXIM1) is an inhibitor of both ERα-positive and -negative breast epithelial cell growth (9). We have previously focused on the role of HEXIM1 as a transcriptional inhibitor of ER. However other studies from our laboratory indicate a role for HEXIM1 as an inhibitor of tumor angiogenesis via downregulation of HIF-1α protein expression and inhibition of HIF-1α mediated transcription (10). Mammary tumors expressing mutant HEXIM1 exhibit increased HIF-1α and VEGF levels and increased vascularization (10). However, how broad an effect HEXIM1 has on HIF-1α regulated gene expression and cellular processes needs to better defined.

Using a well-established model of metastatic breast cancer we observed that targeted re-expression of HEXIM1 in mammary epithelial cells resulted in inhibition of metastasis to the lung. Attenuated metastasis resulting from HEXIM1 re-expression involved decreased vascularization, expression of compensatory angiogenic factors, and recruitment of BMDCs. Further downregulation of HEXIM1 resulted in enhanced migration and invasion of non-invasive breast epithelial cells that can be partly attributed to decreased membrane localization of 67kDa laminin receptor, 67LR.

RESULTS

Expression of HEXIM1 in non-metastatic and metastatic human breast cancers

Our previous studies indicated reduced levels of HEXIM1 expression in breast cancer tissues when compared to normal adjacent breast tissue (9). As an initial test for the potential role of HEXIM1 in metastatic breast cancer we queried the Oncomine database (www.oncomine.org). Seven out of 8 datasets show decreased HEXIM1 expression in primary tumors from patients exhibiting metastatic events when compared to tumors from patients with no metastatic events (Supplementary Figure 1A). The dataset showing no

decrease in HEXIM1 expression consisted only of 3 samples from patients with metastatic events at 1 year compared to 155 samples from patients with no metastatic events (11).

We then examined HEXIM1 expression in 50 pairs of matched primary tumors and metastatic carcinomas in the lymph nodes. We observed a significant decrease in HEXIM1 expression in metastatic carcinomas from the breast relative to the corresponding primary carcinomas (Figure 1A and Supplementary Figure 1B). The mean difference (standard error) was 0.47 (0.17) and the paired t-test for a non-zero mean difference gave a p-value of 0.008.

Expression of HEXIM1 during tumor progression in a well-established model of metastatic breast cancer

The Polyoma Middle-T antigen (PyMT) transgenic mouse is a well-characterized and reliable model of human breast cancer (12). The similarities between PyMT premalignant lesions and many types of human atypical hyperplasias emphasize the value of this model system for preclinical testing. A major underlying reason for selecting the PyMT mouse model for the proposed studies is the activation of the angiogenic switch in these mice (13) that play a role in metastasis to the lung. While other pathways may be involved in the oncogenic process in the PyMT mice, our studies will nevertheless provide important insight into how broad of a protective effect is imparted by HEXIM1 against the dysregulation of other oncogenic pathways. Along this line, microarray analyses of oncogenic initiating events in the PyMT mouse indicate considerable similarities with that of the MMTV-neu, MMTV-ras, and MMTV-myc mouse (14, 15).

We observed decreased HEXIM1 expression in mammary glands from 12 week old PyMT mice when compared to 4 and 8 week old PyMT mice (Figure 1B). Quantification of HEXIM1 staining revealed that HEXIM1 levels in mammary glands from 12 week old mice are 0.35X that of HEXIM1 levels in mammary glands from 8 week old mice (p=0.032). Consistent with our findings in matched human primary and metastatic carcinomas, we observed lower expression of HEXIM1 in lung metastasis than in primary tumors (Figure 1C). Quantification of HEXIM1 staining revealed that HEXIM1 levels in lung metastasis are 0.42X that of HEXIM1 levels in primary tumors (p=0.005) further validating the clinical relevance of this mouse model in the study of the role of HEXIM1 in metastasis.

Re-expression of HEXIM1 through transgene expression inhibits metastasis and angiogenesis

To determine if inducible re-expression of HEXIM1 can inhibit lung metastasis in the PyMT mice, we generated mice that are homozygous for the MMTV-rtTA and pTET-HEXIM1 transgenes (16) and carry at least one PyMT transgene (PyMT/MMTV/HEXIM1 mice) and induced HEXIM1 expression using doxycycline in a tissue specific manner (Supplementary Figure 2). We observed increased HEXIM1 expression in doxycycline treated mice (Figure 2A). Quantification of HEXIM1 staining revealed 2.91 fold increase (p = 0.008) in HEXIM1 levels in mammary glands from doxycycline treated PyMT/MMTV/HEXIM1 mice when compared to control mice. Consistent with previous reports on the PyMT mice (12), the Estrogen Receptor (ER) is expressed at the early stages of tumorigenesis but more advanced carcinomas were ER negative (Supplementary Figure 3), suggesting that some of the effects

of HEXIM1 re-expression on tumor progression may be independent of its effect on ER transcription.

Tumor volumes for each mouse was measured weekly from the start of treatment at 6 weeks through 17 weeks of age (Figure 2B). Based on our analyses, tumors in doxycycline treated PyMT/MMTV/HEXIM1 mice took a predicted 0.74 weeks longer than tumors from control mice to reach the volume corresponding to one half of the (group-specific) maximum (p-value = 0.003). In addition, the estimated maximum log volume and final tumor weights (Supplementary Figure 4) were lower for the doxycycline treated than the control group. However, these differences were not statistically significant.

Re-expression of HEXIM1 in doxycycline treated PyMT/MMTV/HEXIM1 mice also resulted in decreased tumor vascularization (Figures 2C and 2D), tumor proliferation (Figure 2D), and metastasis to the lung (Figures 2C and 2D) when compared to control mice. Re-expression of HEXIM1 resulted in increased apoptosis (Figure 2D).

Re-expression of HEXIM1 via polymer-mediated delivery of HMBA inhibits metastasis and angiogenesis

Alternatively, HEXIM1 expression was induced through HMBA treatment. While HMBA has been shown to cause differentiation of several cancer lines *in vitro* (17), results of *in vivo* studies examining oncolytic activity have been disappointing, showing altered survival patterns, but not an increase in lifespan or number of survivors (18). The dose-limiting toxicity observed in HMBA clinical trials is thrombocytopenia (19) Delivery of HMBA into the mammary gland was achieved using a biodegradable injectable *in situ* forming delivery system comprised of poly(L-lactide-co-glycolide) (PLGA) dissolved in an low toxicity organic solvent, N-methyl pyrrolidone (NMP) (20). PLGA, an FDA-approved polymer, have been utilized in clinical applications such as palliative treatment of prostate cancer (Eligard®) and local delivery of anthracyclines (Atridox®) (20–22).

HMBA was mixed with dissolved PLGA into a final concentration of 5 mM. This loading dose was based on preliminary data indicating that this dose resulted in levels of HMBA within the mammary gland that can induce significant increases in HEXIM1 expression in mammary epithelial cells (Figure 3A, Supplementary Figure 5A). HMBA-loaded PLGA (0.05 cc or 2 mg/kg) was injected into the mammary gland once every 2 weeks, and mammary glands collected 9 weeks after the initial injection. HPLC-MS-MS was used to assess HMBA levels in the mammary glands and sera (Supplementary Figure 5B). We observed about 35-fold lower serum HMBA using PLGA mediated delivery when compared to the mean plasma levels (1 mM) observed in patients after 10-day continuous infusion at 28-day intervals (23). Antitumor effects were observed in some of these patients, however HMBA treatment resulted in dose limiting thrombocytopenia. While decreased platelet levels were evident after systemic exposure of mice to HMBA (Figure 3B), we did not observe evidence of thrombocytopenia (Figure 3B) or weight loss (Supplementary Figure 5C) in PLGA-HMBA injected mice. Note that normal platelet range in mice is 592–2972 K/ul.

Injection of HMBA-PLGA into the mammary gland resulted in decreased tumor volume in HMBA-PLGA treated mice when compared to PLGA treated mice (Figures 3C). Based on our analyses, tumors from HMBA-PLGA treated PyMT mice took a predicted 0.84 weeks longer than tumors from PLGA treated PyMT to reach the volume corresponding to one half of the maximum (p-value = 0.008). The maximums of the two treatment groups and final tumor weights (Supplementary Figure 4) were not statistically significantly different at the 0.05 alpha level. We also observed decreased vascularization of primary tumors and decreased metastasis to the lung in HMBA-PLGA treated mice when compared to PLGA-treated mice (Figures 3D and 3E). Thus PLGA mediated delivery of HMBA resulted in decreased tumor metastasis without the dose limiting toxicity associated with systemic administration of HMBA.

Modulation of proliferative and angiogenic factors by HEXIM1

The phenotypic effects observed with the HEXIM1 transgene is not likely due to repression of the MMTV promoter driving PyMT transgene expression as we did not observe decreased PyMT expression in the mammary glands of doxycycline treated PyMT/MMTV/HEXIM1 mice (Figure 4A). As with HEXIM1 transgene expression, HMBA treatment did not result in decreased PyMT transgene expression in the mammary gland (Figure 4B). With re-expression of HEXIM1 in doxycycline treated PyMT/MMTV/HEXIM1 mice we observed a correlative decrease in cyclin D1, VEGF and HIF-1α expression in mammary tumors from doxycycline treated PyMT/MMTV/HEXIM1 when compared to tumors from control treated mice (Figure 4A). These findings are consistent with our mechanistic studies showing regulation of these factors by HEXIM1 (10, 16). Western blot analyses also indicated increased HEXIM1 expression and decreased cyclin D1, VEGF and HIF-1α expression in mammary tumors from mice injected with PLGA-HMBA when compared to tumors from control (PLGA only injected mice) (Figure 4B).

Antiangiogenic treatments reduce and normalize tumor vasculature but increased intratumor hypoxia eventually develops resulting in hypoxia-mediated induction of proangiogenic factors 1 (5, 6). We observed significantly decreased FGF2 and angiopoietin 1 expression in mammary tumors from doxycycline treated PyMT/MMTV/HEXIM1 mice when compared to tumors from control mice (Figure 4C). Thus HEXIM1 inhibits not only VEGF but also other compensatory angiogenic factors that may contribute to resistance to VEGF inhibitors.

Another way that HIF-1α upregulates angiogenesis is through induction of its target gene SDF-1 that plays a critical role in hypoxia-induced recruitment of BMDCs (24). BMDCs express the SDF-1 receptor CXCR4 and are recruited to the hypoxic tissue by cell tropism to SDF-1. HIF-1α promotes bone metastases by regulating CXCR4, which promotes tumor cell homing to bone (24, 25). Re-expression of HEXIM1 in PyMT/MMTV/HEXIM1 resulted in decreased SDF-1 and CXCR4 levels (Figure 4D). However the decrease in CXCR4 levels may reflect decreased recruitment of BMDCs. Increased HEXIM1 expression did not induce a change in the expression of another HIF-1α target gene, GLUT1, that plays a role in metabolic reprogramming (Figure 4D). Thus regulation of HIF-1α target genes by HEXIM1 is influenced by gene context.

Due to the decrease in SDF-1 and CXCR4 levels we examined the potential for HEXIM1 to regulate recruitment of BMDCs using a marker for BMDCs, in particular the leukocyte lineage marker CD45 and marker for myelomonocytic cells CD11b (26, 27). The presence of $CD45^+$ cells in tumors has been correlated with hypoxia and reported to enhance tumor angiogenesis (27–29). BMDCs $CD11b^+$ myelomonocytic cells, including a subset of $CD11b^+$ $Gr\text{-}1^+$ myeloid derived suppressor (MDSC) cells, enhance tumor angiogenesis, prepare the premetastatic niche in the lung, and are responsible for the refractoriness of tumors to anti-VEGF treatment (30–32). We observed decreased staining for CD45 and CD11b in doxycycline treated PyMT/MMTV/HEXIM1 when compared to tumors from control treated mice (Supplementary Figure 6), suggesting that HEXIM1 re-expression resulted in attenuation of recruitment of BMDCs. Although $CD45^+$ and $CD11b^+$ cells in tumors from doxycycline treated PyMT/MMTV/HEXIM1 mice localized mainly in the perilobular stroma, those in tumors from control mice were able to infiltrate the tumor parenchyma. We verified recruitment of BMDC as playing a role in the inhibition of metastasis by HEXIM1 by collecting tumors at 11 weeks of age, prior to formation of lung metastasis. Flow cytometry analyses indicated increased fraction of $CD11b^+/Gr\text{-}1^+$ cells in both mammary tumors and lungs (Figure 4E).

HEXIM1 inhibits cell invasion

Due to dramatic effects of HEXIM1 on lung metastasis, we examined if HEXIM1 can inhibit other processes, in addition to angiogenesis, critical in metastasis. We observed that down regulation of HEXIM1 expression via introduction of HEXIM1 microRNA (HEXIM1miRNA) into non-invasive breast epithelial MCF7 cells resulted in enhanced invasion of MCF7 cells (Figure 5). Conversely, increased expression of HEXIM1 in MDA-MB-231 cells resulted in attenuation of breast cell invasion (Supplementary Figure 7A).

In most epithelial cancers, loss of the cell-cell adhesion molecule E-cadherin and gain of mesenchymal markers and promigratory signals underlie the conversion of epithelial, differentiated cells to mesenchymal, migratory, and invasive cells, a process referred to as the epithelial-to-mesenchymal transition (EMT) (reviewed in ref. (33)) However, downregulation of HEXIM1 did not result in changes in the levels of E-cadherin in MCF7 cells (Supplementary Figure 7B), suggesting that HEXIM1 regulates other processes, besides EMT, involved in metastasis.

HEXIM1 regulates 67LR localization to the membrane

To determine the mechanistic basis for HEXIM1 inhibition of cell migration and invasion we used yeast two hybrid screening to identify HEXIM1 interacting proteins. One of the factors we have identified that have been reported to play roles in breast cell invasion and/or metastasis is 67LR, a nonintegrin cell-surface receptor with high affinity for laminin (34). 67LR-induced conformational changes result in increased proteolytic cleavage of laminin and consequently generation of proteolytic fragments that promote tumor cell migration (35). 67LR expression has been correlated with enhanced invasive and metastatic potential (36).

We verified the interaction of HEXIM1 with 67LR using endogenous co-immunoprecipitation experiments (Figure 6A). No significant change in total cellular levels of 67LR was evident upon downregulation of HEXIM1 levels using HEXIM1miR (Figure 6B). We then determined if HEXIM1, which is also expressed in the cytoplasm, regulates intracellular localization of endogenous 67LR. Decreased expression of HEXIM1 resulted in increased membrane localization of 67LR (Supplementary Figure 8). The increased 67LR membrane localization upon downregulation of HEXIM1 was verified by increased colocalization of 67LR with E-cadherin in HEXIM1miR-transfected cells (Figure 6C). Conversely, re-expression of HEXIM1 in the PyMT/MMTV/HEXIM1 mice resulted in decreased membrane expression of 67LR, which can also not be accounted for by changes in total 67LR expression (Figure 6D).

Role of 67LR in HEXIM1 inhibition of cell invasion

YIGSR peptide corresponding to laminin sequence specifically bound by 67LR has been shown to compete with laminin and inhibit 67LR mediated laminin conformational change/degradation (37). To determine the relative role of 67LR in the inhibition of cell invasion by HEXIM1 we examined the effect of YIGSR peptide on HEXIM1 regulated cell invasion. Invasion of HEXIM1miRNA cells was attenuated when the YIGSR peptide was introduced into the lower chamber, when compared to HEXIM1miRNA cells exposed to control scrambled peptide (Figure 6E). These findings suggest that the inhibition of 67LR interaction with laminin is important for the ability of HEXIM1 to inhibit invasion.

Microarray profiling supports the role of HEXIM1 in the inhibition of metastasis and regulation of other breast cancer pathways

To further define the molecular basis for HEXIM1 regulation of tumor growth and metastasis we identified genes that are differentially expressed in control miRNA- and HEXIM1miRNA-transfected MCF7 cells using microarray analyses. Pathway analyses of our microarray data support our previous and current findings on HEXIM1 regulated processes and include Estrogen Receptor, VEGF, CXCR4, Angiopoietin, HIF-1α, and chemokine signaling (Supplementary Table 1). Pathways relevant in breast cancer and differentially regulated in control miR- and HEXIM1 miR-transfected cells included p53, TGFβ, JAK/STAT1, BRCA1, HER2, IGF-1 and PI3K/AKT signaling. Metastasis-associated genes that were upregulated as a result of downregulation of HEXIM1 in MCF7 cells and verified by RT-PCR include *TGFB2, ANXA1, LOXL2, and MMP13* (Fig. 7A). Conversely these genes were downregulated in doxycycline treated PyMT/MMTV/HEXIM1 mice when compared to control mice (Fig. 7B). TGFβ signaling is tumor suppressive in normal cells, but once tumors are established TGFβ promotes invasion and metastasis (38). *ANXA1* promotes metastasis formation by enhancing TGFβ/Smad signaling and actin reorganization, resulting efficient cell migration and invasion of breast cancer cells (39). *LOXL2* is a direct transcriptional target of HIF-1α (40) that promotes invasion by regulating the expression and activity of the extracellular proteins tissue inhibitor of metalloproteinase-1 (TIMP1) and matrix metalloproteinase-9 (MMP9) (41). Another metalloproteinase downregulated by HEXIM1, *MMP13*, is important in tumor-induced osteolysis (42).

DISCUSSION

We report on the attenuation of compensatory pro-angiogenic pathways and recruitment of BMDCs as contributing to HEXIM1 inhibition of breast tumor metastasis. Furthermore, HEXIM1 inhibits cell migration and invasion through regulation of 67LR membrane localization. By defining the basis for the anti-metastatic effects of HEXIM1, our studies suggest that re-expression of HEXIM1 in breast cancer have therapeutic advantages by simultaneously targeting more than one pathway involved in metastasis or angiogenesis and thus improve the likelihood of sustained inhibition by blocking the cell's ability to bypass the inhibition of any one pathway. Thus increased HEXIM1 expression has the potential to counteract resistance to therapeutics targeting VEGF. Our studies also support the potential for HEXIM1 to indirectly act on multiple cell type, in particular endothelial cells and BMDCs, to suppress metastatic cancer.

HIF-1α is overexpressed in many human cancers and activates transcription of genes critical in tumorigenesis including angiogenesis, cell survival, glucose metabolism and invasiveness. Thus, HIF-1α represents an attractive target for cancer therapy (43). Similar to what we observed in PyMT/MMTV/HEXIM1 mice, conditional deletion of HIF-1α in the mammary epithelium of PyMT mice resulted in delayed tumor onset, attenuated primary tumor growth, decreased vascularization, and decreased pulmonary metastasis (44). Thus inhibition of HIF-1α expression by HEXIM1 is consistent with the broad effects of HEXIM1 on mammary tumorigenesis.

Antiangiogenic treatments reduce and normalize tumor vasculature. However this eventually results in increased intratumor hypoxia resulting in the production of redundant angiogenic factors by tumors and acquisition of a more invasive phenotype. Late-stage breast cancers express several pro-angiogenic factors, including FGF2, in contrast to earlier stage lesions that preferentially express VEGF (6). Thus, it is possible that pre-existence of FGF2 and the other pro-angiogenic factors in late-stage metastatic tumors enable continuing angiogenesis in the face of bevacizumab therapy, such that inhibition of VEGF signaling does not affect angiogenesis. We now also determined that HEXIM1 inhibits expression of compensatory pro-angiogenic factors. Thus targeting HIF-1α via HEXIM1 re-expression rather than VEGF may offer advantages in late stage breast cancer. We have previously reported on downregulation of HIF-1α protein expression by HEXIM1 (10), and more recent work in our laboratory indicates direct regulation of HIF-1α by HEXIM1 independent of ERα, and implicate HEXIM1 in the inhibition of the progression of ER negative tumors by HEXIM1.

Our findings suggest that HEXIM1 inhibits another compensatory angiogenic processes upregulated by intratumoral hypoxia, the recruitment of BMDCs (24). Current evidence suggests a promotional role of BMDCs on the existing blood vessels rather than de novo neovascularization in tumors. These cells produce different proangiogenic factors and constitute an adaptive mechanism of resistance to angiogenic inhibitors under low oxygen context. Moreover, certain cancer chemotherapeutic drugs inhibit EPC mobilization by inhibiting HIF-1α transcriptional activity (45). BMDCs express the SDF-1 receptor CXCR4 and are recruited to hypoxic tissue by cell tropism to SDF-1 (46). VEGF, SDF-1 and CXCR4 are all HIF-1α target genes and studies indicate that HIF-1α is required for

endothelial progenitor cell (EPC) colony formation, differentiation, proliferation and migration (47–49). However further studies examining the recruitment of $VEGFR1^+$ hematopoietic precursor cells and $VEGFR2^+$ endothelial precursor cells are necessary to show that the inhibition of recruitment of BMDCs are involved in the attenuation of tumor angiogenesis by HEXIM1.

HEXIM1 regulation of recruitment of BMDCs may also play a role in its ability to inhibit breast cancer metastasis. Although tumor cells are the driving force of metastasis, findings suggest that the host cells within the tumor microenvironment play key roles in influencing metastatic behavior (reviewed in ref. (50)). Recruited bone marrow progenitor cells generate a “pre-metastatic niche” to which the tumor cells metastasize. Analysis of the molecular mechanisms involved in pre-metastatic niche formation has revealed that secreted soluble factors, such as VEGF, SDF-1, LOXL2, TGFβ are key players in bone marrow cell mobilization during metastasis (50). HEXIM1 inhibition of the expression of these factors is a potential molecular basis for HEXIM1 inhibition of metastasis.

Like HIF-1α, 67LR is involved in the recruitment of BMDCs. Human bone marrow have been shown to be rich in laminin (51, 52) and 67LR contributes to the egress of stem cells from the bone marrow by mediating laminin-dependent cell adhesion and transendothelial migration (53). Our studies indicate that HEXIM1 inhibits membrane localization of 67LR. 67LR overexpression is considered a molecular marker of metastatic aggressiveness in cancers of many tissues (54). 67LR promotes tumor cell migration and adhesion (35) chemotaxis, and migration through vessel walls (55), and increases extracellular matrix degradation by up-regulating the expression and activity of proteolytic enzymes (37). Laminin conformation changes upon binding 67LR, allowing for more efficient interaction with integrin and enhanced sensitivity to the action of proteolytic enzymes (56). Cytoplasmic HEXIM1 may inhibit 67LR activity by simply attenuating 67LR membrane localization by the interaction between HEXIM1 and 67LR. 67LR derives from a 37-kDa cytosolic precursor (37LRP) that when post-translationally modified by acetylation, forms dimers of approximately 67 kDa that are expressed on the surface of cells (57). While it is possible that HEXIM1 regulates formation of 67LR form its 37LRP precursor, we did not observe alterations in total 67LR protein levels upon downregulation of HEXIM1 expression.

While it is likely that HEXIM1 is not the only mediator of the oncolytic effects of HMBA, this should not detract too much from its potential as a breast cancer therapeutic. The continuing development of “targeted agents” against different forms of cancers provides important lessons for the future development of cancer therapy. Most notable is the fact that any given (small molecule) therapeutic agent will have other targets within a cancer cell that were not originally conceived of (58) and that this is not necessarily a negative with regards to potential clinical utility. The first wave of ‘targeted’ agents exemplified by imatinib (Gleevec®) has been found to hit a range of targets, despite being originally developed as a selective agent. We obtained promising new results on the ability of HMBA to inhibit metastasis. HMBA has been known to be a potent differentiating agent for some time. Loss of differentiation is one of the hallmarks of malignancy, and the interest in differentiation therapy is based on the rationale that resuming the process of maturation in undifferentiated

cancer cells compromises their proliferative capacity. While attenuation of metastasis to the lung in our studies can be attributed to HMBA-induced HEXIM1 expression and angiogenesis in primary tumors cells, induction of differentiation through HEXIM1 and/or other targets and HMBA in the circulation acting on circulating tumor cells may also be contributing factors. Overall, our studies provide support for studies examining the direct targets of HMBA in breast cells that results in upregulation of HEXIM1 mRNA expression, in addition to the development of HMBA derivatives.

METHODS

Mouse models

All animal work reported herein have been approved by the CWRU Institutional Animal Care and Use Committee. All mice in this study were generated using FVB mouse strain. HEXIM1 expression was induced in mammary epithelial cells of PyMT mice by mating MMTV/HEXIM1 bitransgenic mice (16) with PyMT mice. Mice were genotyped as described in the Supplementary section. HEXIM1 expression was induced by supplementing the drinking water of mice with doxycycline at a final concentration of 2 mg/ml.

Production of PLGA ± HMBA is described in the Supplementary section. For HMBA studies, 50 ul of PLGA or PLGA-HMBA were injected into the left thoracic mammary glands of PyMT mice, every other week starting at 6 weeks of age. Tumor latency was determined as the first day a tumor was palpated. To allow for detection of metastasis to the lung, PyMT mice that were treated with PLGA or PLGA-HMBA were sacrificed at 15 weeks of age, and MMTV/PyMT/HEXIM1 mice (± doxycycline) were sacrificed at 17 weeks of age.

To limit variability, each control mouse was from the same litter as the corresponding doxycycline treated PyMT/MMTV/HEXIM1 or HMBA-PLGA treated PyMT mouse. For all comparisons the value for the control mouse in the pair is set at 1 and the relative change in the mammary glands of the treated mouse was expressed relative to this value.

Immunohistochemistry

Tissue array containing sections of human primary tumors and matching lymph node metastasis were obtained from U.S. Biomax (Rockville, MD). We carried out immunohistochemical staining to detect HEXIM1 levels as previously described (59). Staining was classified according to intensity of HEXIM1 expression in tumor cells in 4 categories: negative (0), weak (1), moderate (2), strong (3), and very strong (4) as previously described (59).

The left thoracic mammary tumors from PyMT mice or the largest tumor from MMTV/PyMT/HEXIM1 mice were collected and divided into 3 parts for western blots, paraffin sections and frozen sections. Lungs were also collected, fixed, embedded in paraffin for hematoxylin and eosin (H&E) staining to assess metastasis

Tissue sections were fixed and stained as previously described (10) and details are provided in the Supplementary section.

Flow cytometry analyses of CD11b$^+$/Gr1$^+$ cells in mammary tumors and lungs

Single cells suspensions from mammary tumors and lungs were preblocked with Fc block (CD16/CD32; eBioscience) and then stained using the murine CD11b APC antibody (to stain for monocytes/macrophages, eBioscience) and Gr-1 FITC antibody (to stain for granulocytes, eBioscience). Stained cells were measured on a FACsAria flow cytometer.

HPLC/MS/MS

HMBA in mouse sera was prepared and analyzed using a published method (60). The method was modified and validated for analysis of HMBA in tissues as described in the Supplementary section.

Western Analysis

Tissue and cell lysates were analyzed by western blot as previously described (16).

Yeast two hybrid

The yeast two-hybrid screenings used to identify HEXIM1-interacting proteins were described previously (61) except pBD-GAL-HEXIM1 (9) was used as a bait..

Cell culture

MCF7-control miRNA and MCF7-HEXIM1 miRNA cells were generated as previously described (16). MDA-MB-231 cells were transfected with control vector or expression vector for Flag-HEXIM1 as previously described (10).

Coimmunoprecipitation

Endogenous proteins were co-immunoprecipitated and analyzed as previously described (62). Details are provided in the Supplementary section.

Invasion assays

Details are provided in the Supplementary section.

Immunofluorescent staining

Cells were stained as previously described (9). Details are provided in the Supplementary section.

Microarray analyses

Details are provided in the Supplementary section.

Reverse Transcription (RT) PCR Analyses

MCF7-control miRNA and -HEXIM1 miRNA cells and mammary tissue from 11-week old MMTV/PyMT/HEXIM1 mice (± doxycycline) were subjected to RT-PCR analyses as previously described (16). Primer sequences are provided in the Supplementary section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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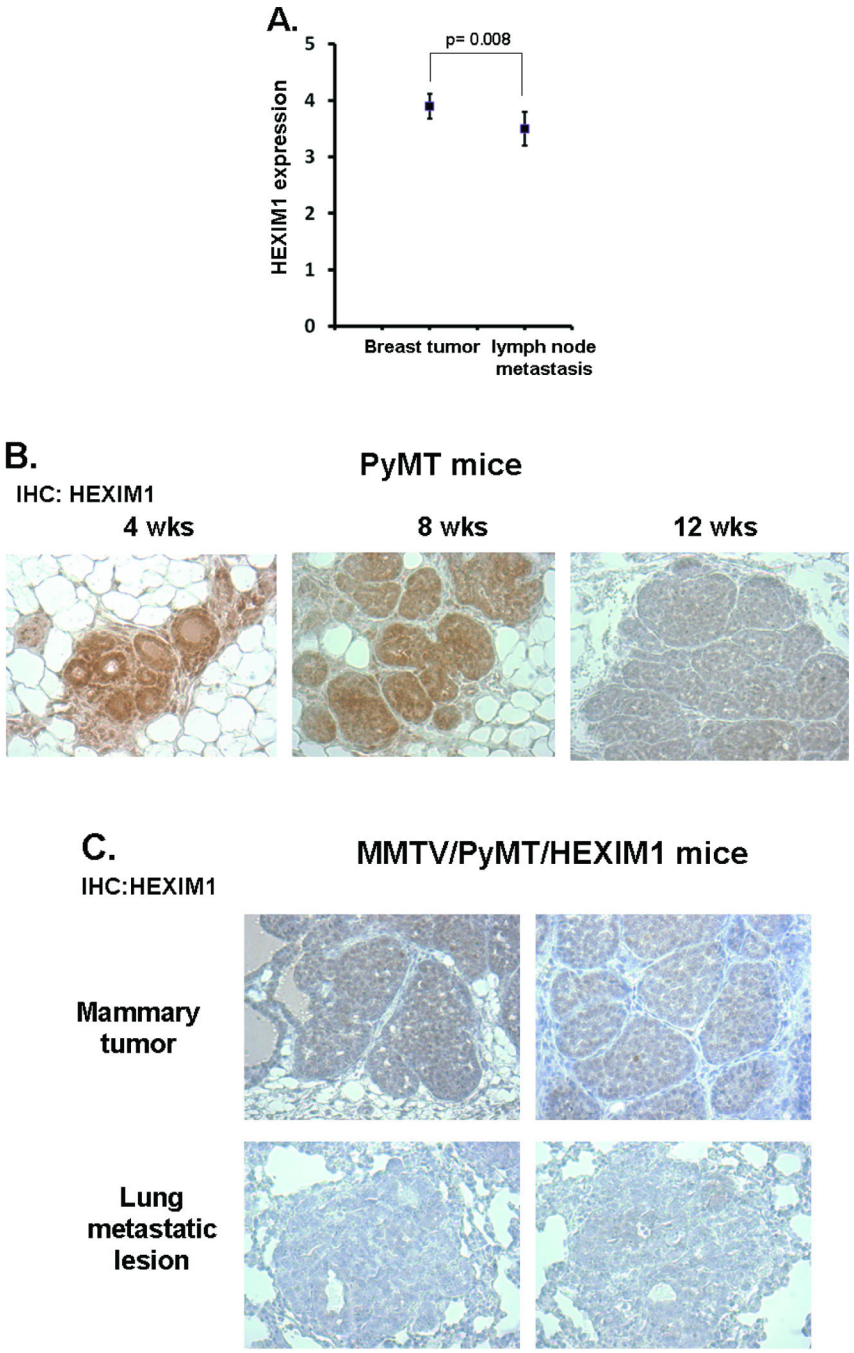

Figure 1. Expression of HEXIM1 in primary and metastatic mammary cancer

(A) Sections from 50 pairs of human primary tumors and matching lymph node metastasis were stained for endogenous HEXIM1. Scores were between 1 and 5 (higher score indicating greater intensity and percent of cells stained). Mean HEXIM1 staining and the standard error were calculated for each tissue type. A difference score (tumor minus lymph) was calculated for each paired samples, and the mean and standard error were calculated for the sample. A paired t-test was performed to test the null hypothesis that the mean difference score is equal to zero. **(B)** Immunohistochemical detection of HEXIM1 in the mammary

gland (4, 8, 12 weeks) and **(C)** paired primary mammary tumors and lung metastasis from PyMT mice.

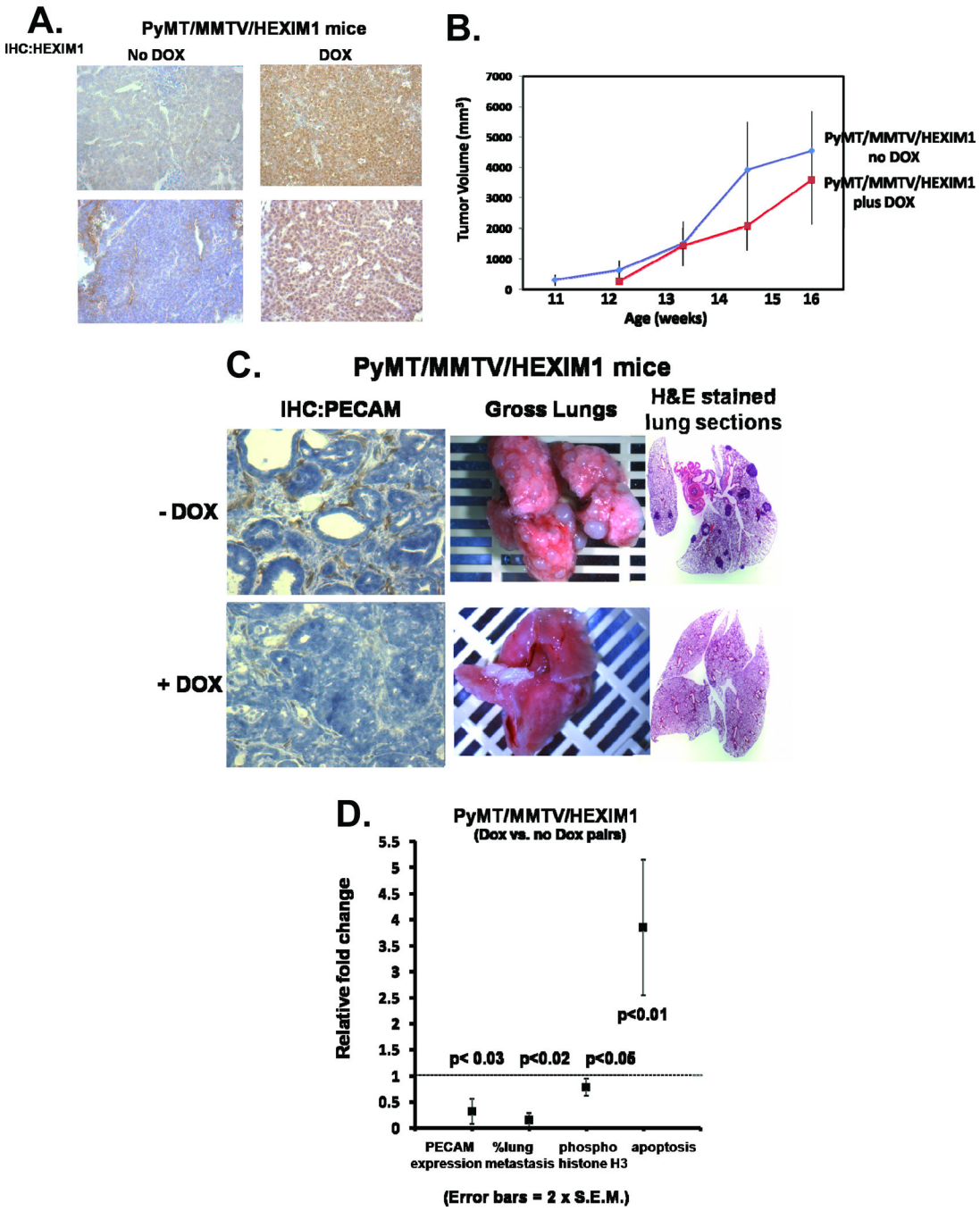

Figure 2. HEXIM1 transgene expression resulteds in decreased mammary tumor volumes, vascularization, and metastasis to the lung

PyMT/MMTV/HEXIM1 mice were treated with doxycycline at 6 weeks of age and tumor volume monitored until 17 weeks of age. Mammary glands and lungs were then collected and processed for histology and immunohistochemistry or western blot analyses. **(A)** Immunohistochemical detection of HEXIM1 in the mammary glands of control and doxycycline treated PyMT/MMTV/HEXIM1 mice. **(B)** Mammary tumor volumes from control (n=12) and doxycycline treated PyMT/MMTV/HEXIM1 (n=15) mice. **(C)** Left

panel: Immunohistochemical detection of PECAM-1 in mammary tumors from control and doxycycline treated PyMT/MMTV/HEXIM1 mice. Center and right panels: Lungs from control and doxycycline treated PyMT/MMTV/HEXIM1 mice. **(D)** Quantification of % PECAM-1-positive staining area in tumor sections, and % tumor area in H&E stained lung tissues, phospho histone H3 (mitotic marker) expression determined using western blot analyses, and % apoptotic cells determined using TUNEL staining. Bars represent mean ± 2 S.E.M. from at least 8 mice per group (± doxycycline). For (C) and (D) control and treated mouse pairs were from the same litter to limit variability and for all comparisons the value for the control mouse in the pair is set at 1 and the relative change in the mammary gland of the treated mouse was expressed relative to this value.

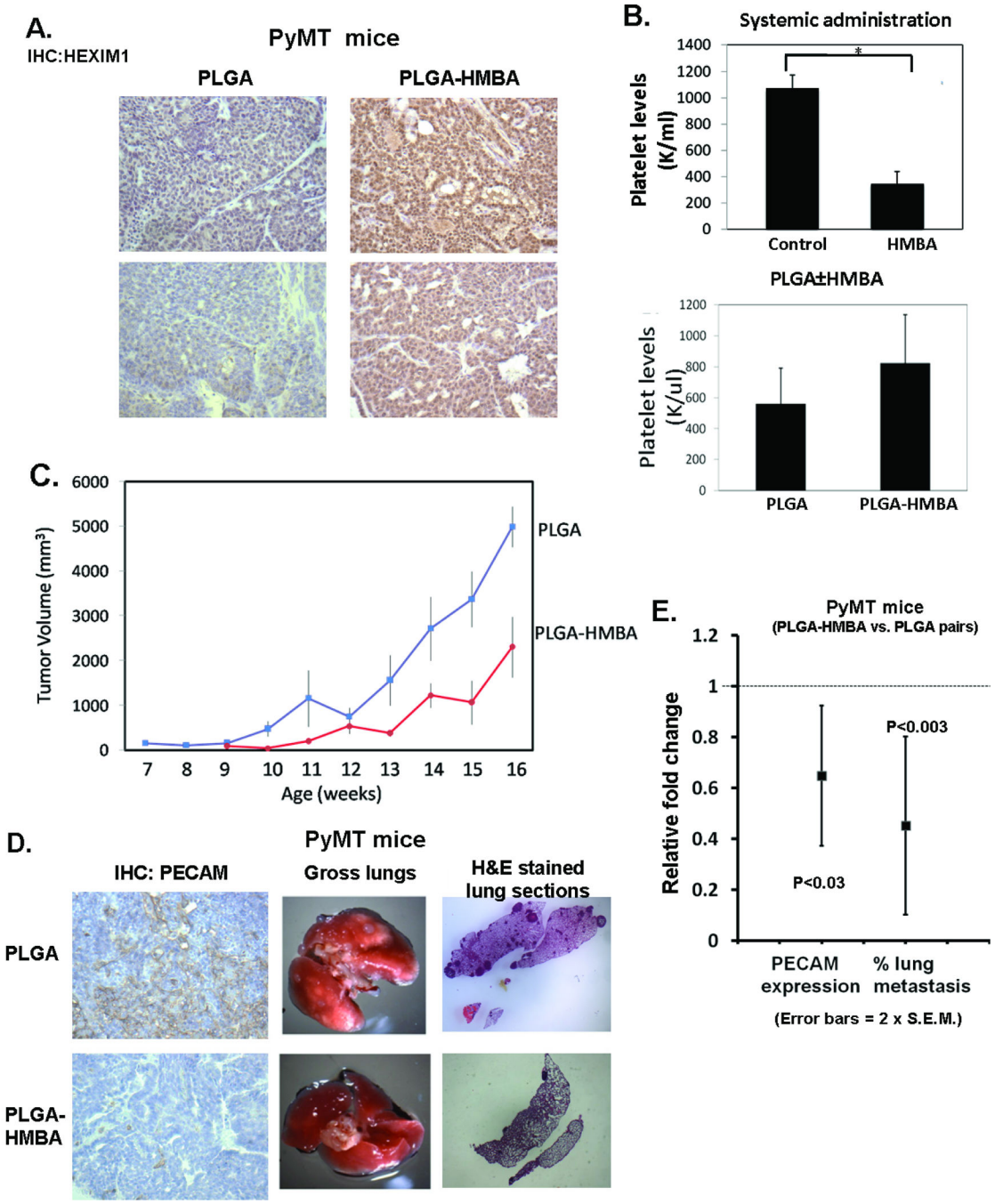

Figure 3. Injection of HMBA-PLGA resulteds in decreased mammary tumor volumes, vascularization, and metastasis to the lungs

PyMT mice were injected with PLGA or PLGA-HMBA (2 mg/kg) into the left thoracic mammary glands of PyMT mice, every other week starting at 6 weeks of age. Mice were monitored until 15 weeks of age, after which mammary glands, blood, and lungs were collected. **(A)** Immunohistochemical detection of HEXIM1 in the mammary glands of PLGA ± HMBA treated PyMT mice. **(B)** Platelet levels were determined using the HEMAVET 950FS Multi-species Hematology System. **(C)** Mammary tumor volumes after

injection with PLGA ± HMBA. **(D)** Left panel: Immunohistochemical detection of PECAM-1 in mammary tumors from PLGA or HMBA-PLGA treated PyMT mice. Center and right panels: Lungs from PLGA or HMBA-PLGA treated PyMT mice. **(E)** Quantification of % PECAM-1-positive staining area tumor sections and % tumor area in H&E stained lung tissue sections. Bars represent mean ± 2 S.E.M. from at least 15 mice per group (PLGA ± HMBA). For (D) and (E) control and treated mouse pairs were from the same litter to limit variability and for all comparisons the value for the control mouse in the pair is set at 1 and the relative change in the mammary gland of the treated mouse was expressed relative to this value.

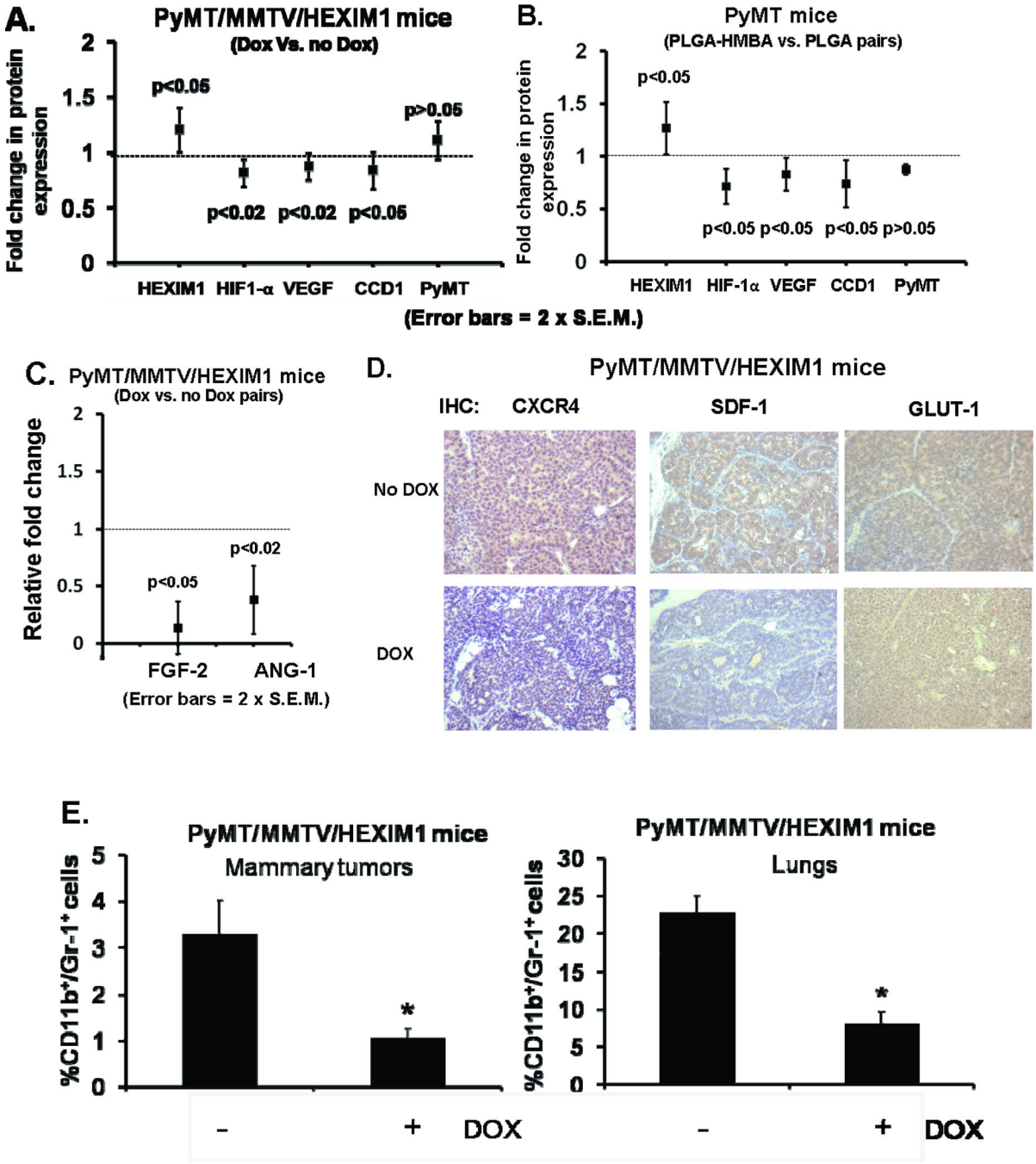

Figure 4. Modulation of proliferative and angiogenic factors and recruitment of BMDCs by HEXIM1

(A) Western blot analyses of HEXIM1, HIF-1α, VEGF, cyclin D1, and PyMT expression in tumor lysates from control and doxycycline treated PyMT/MMTV/HEXIM1 mice. Blots were probed for β-actin as a loading control. Bars represent mean ± 2 S.E.M. from at least 7 mice per group (± Dox). **(B)** Western blot analyses of HEXIM1, HIF-1α, VEGF, cyclin D1, and PyMT expression in tumor lysates from PLGA or HMBA-PLGA treated PyMT mice. Blots were probed for β-actin as a loading control. Bars represent mean ± 2 S.E.M. from at

least 8 mice per group (± HMBA). **(C)** Quantitation of immunostaining of mammary tumors from control and doxycycline treated PyMT/MMTV/HEXIM1 mice (n=4 pairs) for compensatory angiogenic factors FGF2 and angiopoietin 1. **(D)** Immunohistochemical detection of other HIF-1α target genes, SDF-1, CXCR4, and GLUT1. **(E)** and markers for BMDCs, CD45 andFlow cytometry analyses of $CD11b^{+}/Gr\text{-}1^{+}$ cells CD11b, in mammary tumors and lungs from 11 week old control and doxycycline treated PyMT/MMTV/HEXIM1 mice (± Dox). Panels represent immunostaining values from 3 pairs of PyMT/MMTV/HEXIM1 mice (± Dox).

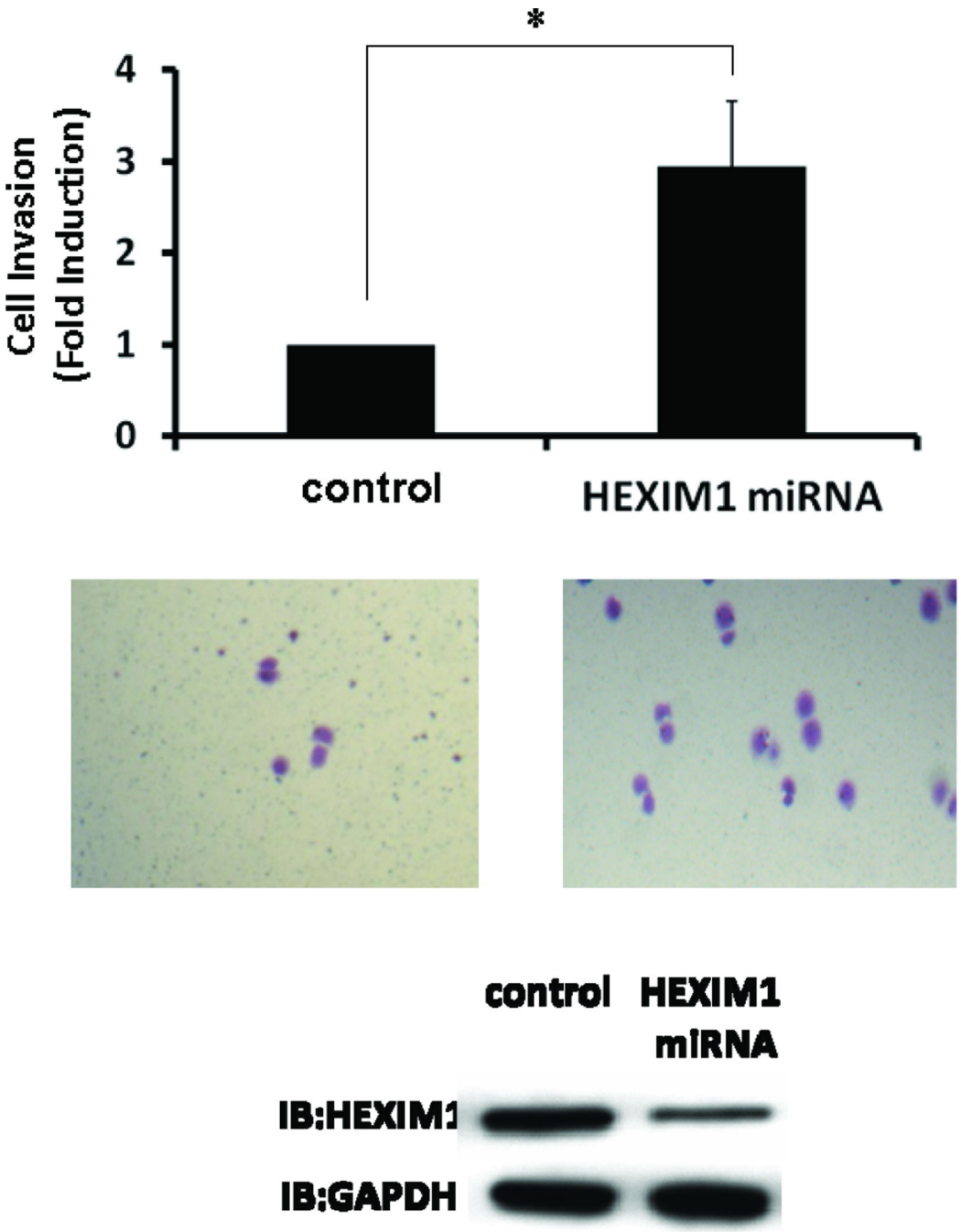

Figure 5. Downregulation of HEXIM1 resulted in enhanced invasion of MCF7 cells

Invasion of MCF7-control miRNA and -HEXIM1 miRNA cells through Matrigel in the transwell invasion assay. Bottom panel: Images of invaded cells that are stained with crystal violet for visualization. Each column or image represent the mean of three replicates; bars, SE. *, $P < 0.01$.

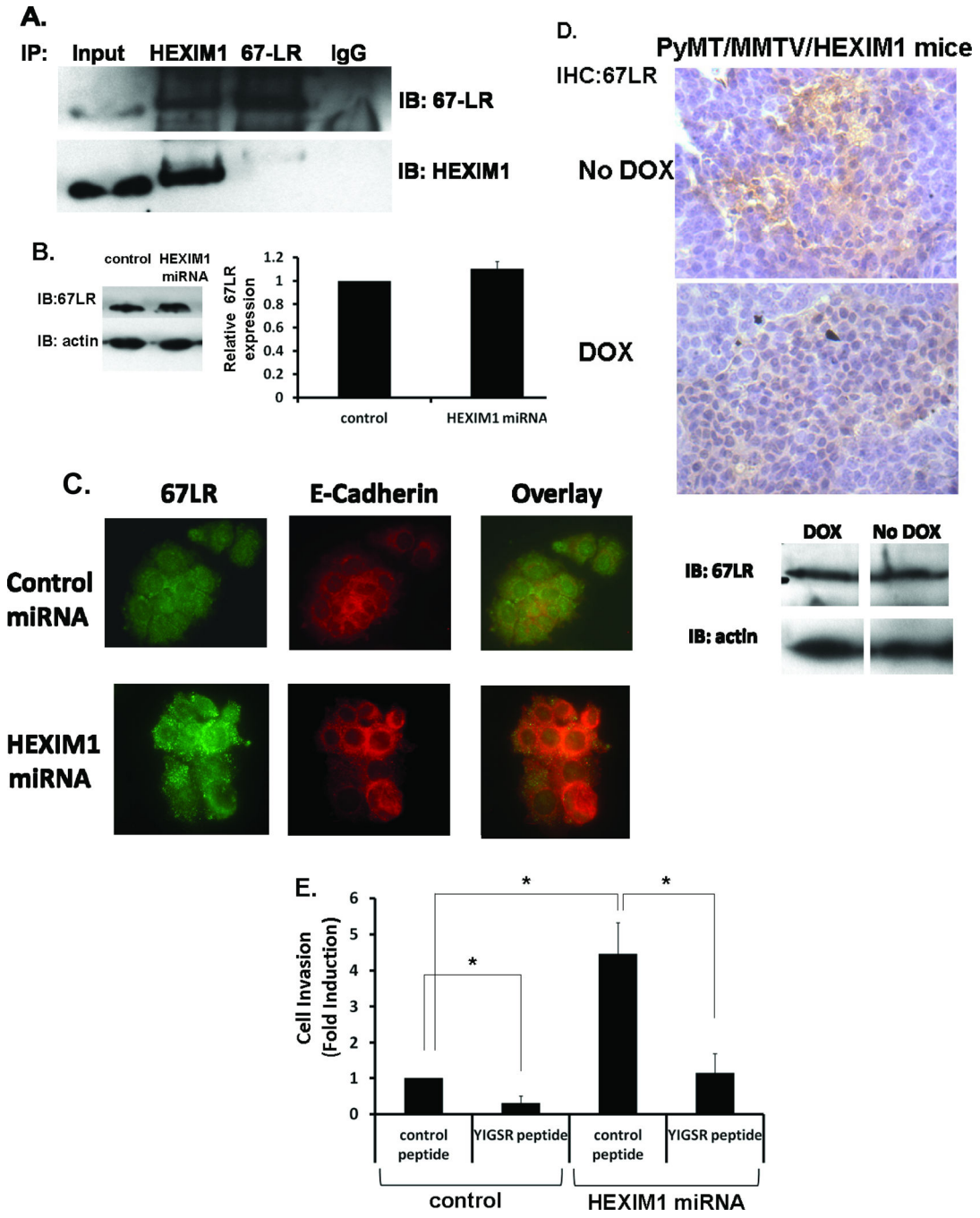

Figure 6. Inhibition of 67LR interaction with laminin is important for the ability of HEXIM1 to inhibit invasion

(A) Lysates from MCF7-control miRNA and -HEXIM1 miRNA cells were immunoprecipitated using antibodies against 67LR or HEXIM1 and analyzed for co-immunoprecipitating proteins by Western blotting using 67LR antibody. Normal rabbit immunoglobilin was used as a specificity control. Input lanes represent 25% of the total protein. **(B)** Western blot analyses of 67LR expression in MCF7-control miRNA and -HEXIM1 miRNA cells. **(C)** MCF7-control miRNA and -HEXIM1 miRNA MCF7 cells

were stained for 67LR and E-cadherin as described in the Supplementary Methods. Images are representative of three replicate experiments. **(D)** Immunohistochemical detection and Western blot of 67LR in the mammary glands of control and doxycycline treated PyMT/MMTV/HEXIM1 mice. Images represent experiments with at least 5 pairs of PyMT/MMTV/HEXIM1 mice (± Dox). **(E)** Invasion of MCF7- control miRNA and -HEXIM1 miRNA cells through Matrigel in the transwell invasion assay was assessed in the presence of control scrambled or YIGSR peptide in the lower chamber. Each column or image represent the mean of three replicates; bars, SE. *, $P < 0.01$.

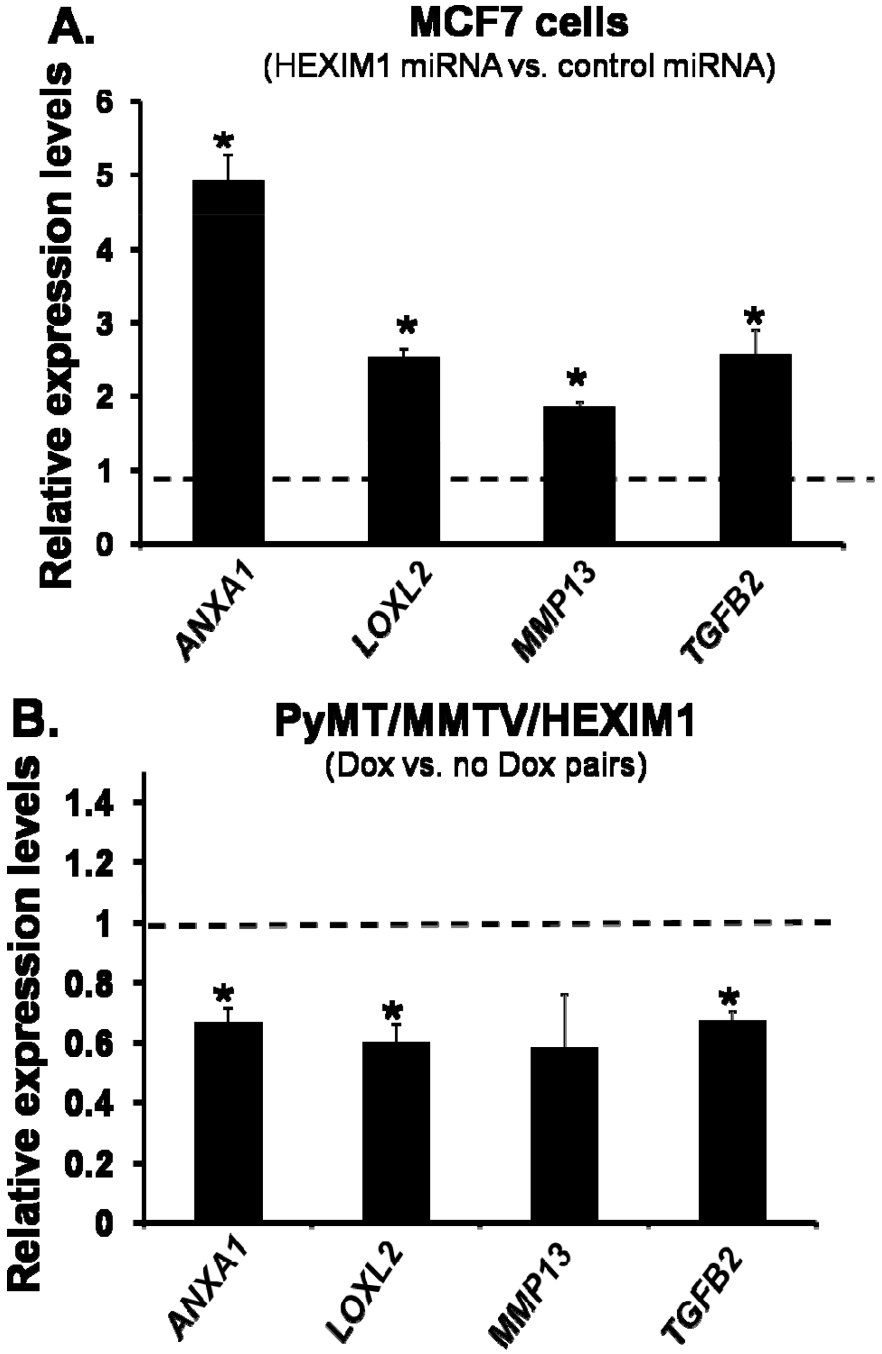

Figure 7. HEXIM1 regulated expression of other metastasis associated genes

Expression levels of metastasis associated genes in **(A)** MCF7-control miRNA and -HEXIM1 miRNA cells and **(B)** 11-week old MMTV/PyMT/HEXIM1 mice (± Dox) identified using microarray analyses and confirmed using RT-PCR. For all comparisons the value for the control miRNA cells or control MMTV/PyMT/HEXIM1 mouse is set at 1 and the relative change in the HEXIM1 miRNA cells or Dox-treated MMTV/PyMT/HEXIM1

mouse was expressed relative to this value. Each column or image represent the mean of three replicates; bars, SE. *, $P < 0.01$.