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Co-Expression of VEGF and IL-6 Family Cytokines is Associated with Decreased Survival in HER2 Negative Breast Cancer Patients: Subtype-Specific IL-6 Family Cytokine-Mediated VEGF Secretion[1,2]

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Abstract

Breast cancer cell-response to inflammatory cytokines such as interleukin-6 (IL-6) and oncostatin M (OSM) may affect the course of clinical disease in a cancer subtype-dependent manner. Furthermore, vascular endothelial growth factor A (VEGF) secretion induced by IL-6 and OSM may also be subtype-dependent. Utilizing datasets from Oncomine, we show that poor survival of invasive ductal carcinoma (IDC) breast cancer patients is correlated with both high VEGF expression and high cytokine or cytokine receptor expression in tumors. Importantly, epidermal growth factor receptor-negative (HER2-), but not HER2-positive (HER2+), patient survival is significantly lower with high tumor co-expression of VEGF and OSM, OSMRβ, IL-6, or IL-6Rα compared to low co-expression. Furthermore, assessment of HER2- breast cancer cells *in vitro* identified unique signaling differences regulating cytokine-induced VEGF secretion. The levels of VEGF secretion were analyzed by ELISA with siRNAs for hypoxia inducible factor 1 α (HIF1α) and signal transducer and activator of transcription 3 (STAT3). Specifically, we found that estrogen receptor-negative (ER-) MDA-MB-231 cells respond only to OSM through STAT3 signaling, while ER+ T47D cells respond to both OSM and IL-6, though to IL-6 to a lesser extent. Additionally, in the ER+ T47D cells, OSM signals through both STAT3 and HIF1α. These results highlight that the survival of breast cancer patients with high co-expression of VEGF and IL-6 family cytokines is dependent on breast cancer subtype. Thus, the heterogeneity of human breast cancer in relation to IL-6 family cytokines and VEGF may have important implications in clinical treatment options, disease progression, and ultimately patient prognosis.

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Introduction

In the United States, breast cancer is the most frequently diagnosed cancer among women [1]. With 268,670 new cases of invasive breast cancer (IBC) expected for 2018, breast cancer remains a leading public health concern, both in the United States and globally. One of the main concerns is the complex relationship between IBC subtypes, clinical treatment, and long-term survival [1,2]. In particular, anti-angiogenic treatments for breast cancer have had variable clinical success at best, and at worst, show no improvement in disease-free survival [3]. The high level of clinical variability with anti-angiogenic therapies may be due, in part, to the highly heterogeneous nature of breast cancer and their subsequent biomarkers [4]. This underlies

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the need to improve our understanding of clinical outcomes in conjunction with existing breast cancer subtype biomarkers such as estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor 2 (HER2).

Current breast cancer subtype classifications depend on the evaluation of ER, PR, and HER2 [5]. The luminal A (ER+ PR+ HER2-; low Ki67) breast cancer subtype is the least aggressive, while the luminal B (ER+ PR+ HER2-; high Ki67 or HER2+) breast cancer subtype is more aggressive and has an overall poorer prognosis [5–7]. In contrast, cancers classified as basal-like triple negative breast cancer (TNBC; ER- PR- HER2-) are highly aggressive with increased probability of relapse and display unfavorable prognoses, in large part due to the lack of targeted therapies available to treat this subtype [4,8]. Similarly, HER2-type breast cancers (ER- PR- HER2+) also have poor prognoses [9]. While superficial differences between the subtypes are relatively well known, the specific molecular mechanisms that drive these differences remain elusive. Specifically, increased inflammatory markers in the serum of breast cancer patients appear to be associated with poor prognosis [10].

Inflammatory cytokines of the interleukin-6 (IL-6) family, including IL-6 and oncostatin M (OSM), have been implicated in the migration and invasiveness of human breast cancer cells [11–13], while leukemia inhibitory factor (LIF) has been shown to act as a tumor/metastasis suppressor [14–17]. Moreover, both IL-6 and OSM have been shown to be capable of directly and indirectly driving angiogenesis [18–20]. Receptor complexes of the IL-6 family all consist of at least one molecule of glycoprotein 130 (gp130), as well as subunit(s) specific to their respective receptor [21,22]. Following IL-6 or OSM ligand binding, the gp130 receptor complexes activate the JAK/STAT, MAPK, PI3K/AKT, and JNK pathways, thereby mediating transcription of target genes [22–27]. Although IL-6 and LIF bind specifically to their individual receptors (IL-6R and LIFR, respectively), OSM is capable of binding to both the LIFR and the OSM receptor (OSMR), which it binds to with higher affinity [28–30]. The resultant cytokine signaling cascades play various roles in the progression of breast cancer through activation of target genes involved in differentiation, survival, apoptosis, and angiogenesis [26,31,32].

Normal angiogenesis is maintained in homeostasis by numerous pro- and anti-angiogenic factors, resulting in a normal rate of blood vessel growth [33]. During tumor angiogenesis, both tumor cells and tumor-associated stromal/immune cells secrete proangiogenic factors [27,34]. The most potent proangiogenic factor, vascular endothelial growth factor A (VEGF), promotes the survival, proliferation, and motility of endothelial cells and enhances vascular permeability [35,36]. Regulation of VEGF expression typically depends on hypoxia-driven signaling through the binding of the dimeric transcription factor hypoxia-inducible factor 1 (HIF1) [HIF1α + HIF1β] to the hypoxia response element (HRE) in the VEGF promoter. [37]. However, activation of transcription factor binding to sites other than the HRE, such as those for signal transducer and activator of transcription-3 (STAT3), allow transcription to be activated independently of hypoxia [38–41]. While it has been shown that hypoxia can induce STAT3 phosphorylation [42,43], hypoxia-induced pSTAT3 is not rapid. Therefore, cellular hypoxia likely promotes the secretion of cytokines from breast tumor cells that then promote STAT3 phosphorylation. In fact, hypoxia has been shown to increase production of cytokines such as IL-6, IL-1α, and IL-1β, which are all known to activate STAT3 signaling [44,45]. Even under non-hypoxic conditions, IL-6 family cytokines have been shown to promote VEGF expression *via* activation of transcription factors HIF1α and STAT3 [19,27,46,47].

In this paper, we study the differential effects of the inflammatory interleukin-6 (IL-6)-family cytokines on breast cancer patient outcomes, as well as the induction of vascular endothelial growth factor A (VEGF) in a breast cancer subtype-specific manner. Using collated Oncomine data, we systemically explored the co-expression of VEGF with inflammatory cytokine components among invasive ductal carcinoma (IDC) patients subdivided by HER2- or HER2+ status. Importantly, we found that HER2- patient survival significantly decreases when breast tumors co-express high levels of VEGF and high levels of OSM, OSMRβ, IL-6, or IL-6Rα. Conversely, in patients with HER2+ disease, co-expression of VEGF and these inflammatory proteins had little to no effect on survival. We also show that regulation of IL-6-family cytokine-induced VEGF in HER2- cells differ between ER+ and ER- breast cancer cells. This study highlights the breast cancer subtype-specific differences in cytokine signaling that lead to VEGF secretion, and importantly, the potential for therapeutic suppression of IL-6 family cytokines in HER2- breast cancer.

Materials and Methods

Oncomine Analysis

We utilized the Curtis Breast human mRNA microarray dataset from Oncomine (Compendia Bioscience, Ann Arbor, MI) to assess correlations between inflammatory cytokines and VEGF. The constraints used to define the dataset used were "Invasive Ductal Carcinoma" and a detailed survival status of either "Alive" or "Dead of Disease." The resultant dataset was used to calculate quartiles. From these, the upper quartile (>75th percentile) and lower quartile (<25th percentile) were selected for comparison in order to clearly depict survival trends that may have been otherwise muddled by use of all quartile combinations. For co-expression analysis, we calculated survival curves using patients in the upper quartile of both VEGF and each particular IL-6 family gene ("high/high") and the lower quartile of both ("low/low"). Statistical analyses between survival of two groups was calculated using a log-rank test in GraphPad Prism 5 software $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

Tissue Culture

Triple negative MDA-MB-231, luminal A T47D, and triple positive BT474 human breast cancer cells (ATCC) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin, and 1% sodium pyruvate and incubated at 37 °C at 5% CO_2. HER2+ SK-BR-3 human breast cancer cells were grown in McCoy's 5A medium, while MDA-MB-453 human breast cancer cells were grown in DMEM with the same supplements as above. Cytokine treatments, in low serum media (1% FBS) at 25 ng/mL, included recombinant human OSM (Cat#300-10 T, Peprotech), IL-6 (Cat #200–06, Peprotech), and LIF (Cat# 300–05, Peprotech) for the indicated time points.

siRNA Transfection

siRNA pools targeting HIF1α, STAT3, JNK1, and JNK2 were obtained from Dharmacon. In brief, 300,000 cells/well were plated in a 6-well plate, and siRNAs were transfected according to the Fast-Forward protocol as outlined by the manufacturer of Hyperfect siRNA Transfection Reagent (Cat# 301705, Qiagen). STAT3 siRNAs were used at 25 nM, and cells were transfected for 72 hours and then treated with OSM for 48 hours. HIF1α, JNK1, and JNK2 siRNAs were used at concentrations of 20 nM, and were transfected for 24 hours prior to OSM treatment. Knockdown was assessed *via* immunoblot analysis (see below).

Immunoblot Analysis

Cells were lysed on ice with 1x RIPA buffer containing 1x protease inhibitor cocktail (Cat# P8340, Sigma Aldrich). Lysates were run on an SDS-PAGE gel, transferred onto a nitrocellulose membrane, and blocked overnight in 5% non-fat dry milk in PBS containing 0.05% Tween 20. Membranes were incubated overnight with primary antibodies for HIF1α (Dilution: 1:1000 Cat# AF1935, R&D Systems), STAT3 (Cat# 9132), p-STAT3 (Cat# 9145), JNK1 (Cat# 3708), p-JNK1 (Cat# 4668), or β-Actin (Cat# 3700) ((1:1000, Cell Signaling Technologies) in 5% NFDM-PBST. Membranes were washed with PBST and incubated with HRP secondary antibody (Cat# 705–035-003 Jackson ImmunoResearch) in 5% NFDM-PBST for 45 minutes, developed with ECL, and imaged on X-ray film on a Kodak 4000R Image Station.

VEGF ELISA

Analysis of VEGF secretion in the conditioned media (CM) of MDA-MB-231, T47D, MCF7, SK-BR-3, MDA-MB-453, and BT474 (ATCC, Manassas, VA) breast cancer cells was assessed *via* VEGF ELISA according to the manufacturer's protocol (Cat# Dy293B, R&D Systems, Bethesda, MD). Cells were plated at a confluency of 50,000 or 100,000 cells in a 24-well plate and allowed to adhere overnight. The following day, cells were serum-starved in serum free media for 6 hours and treated with cytokines for the indicated time.

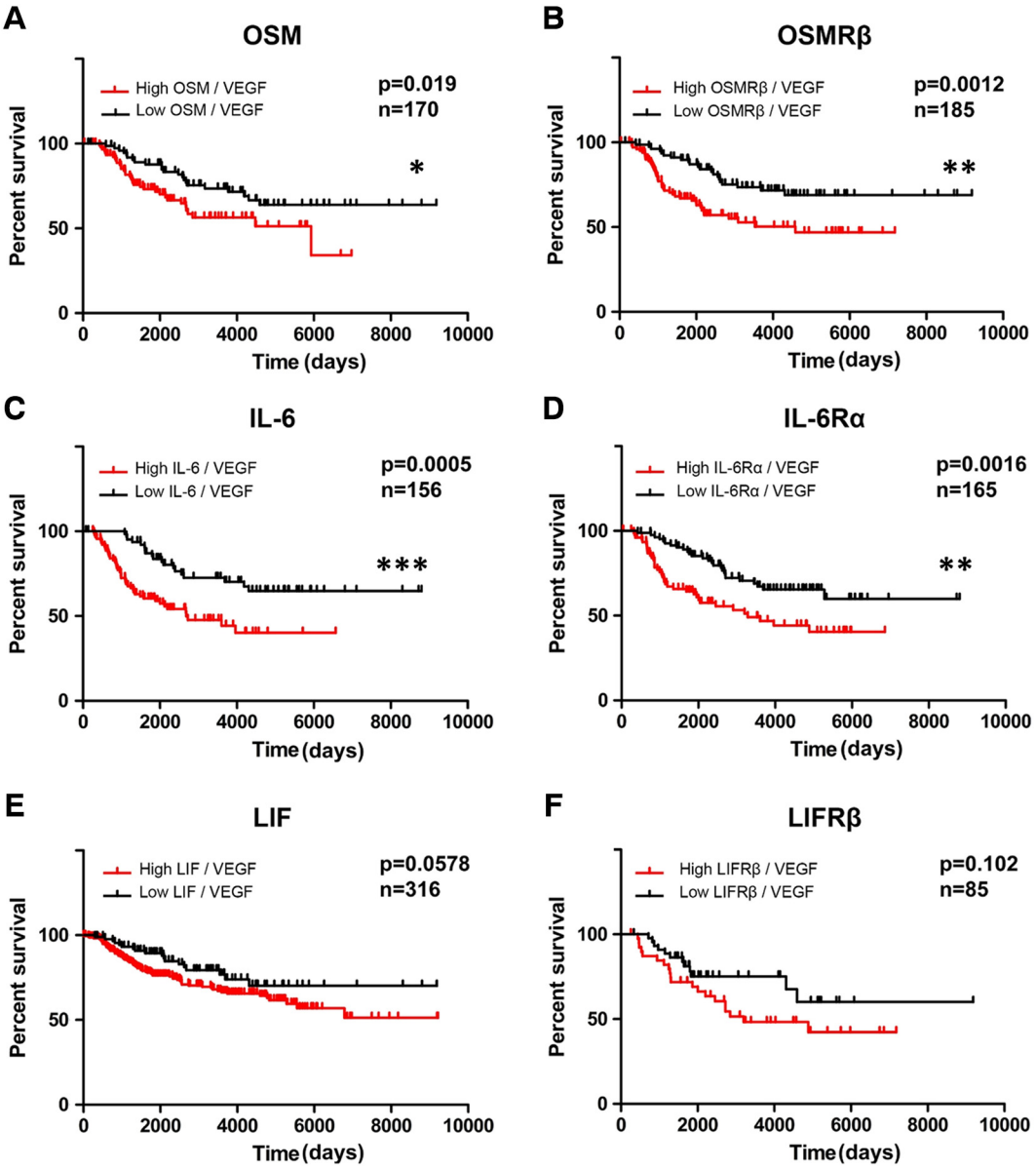

Figure 1. Inflammatory cytokine and VEGF co-expression are correlated with decreased survival of invasive ductal carcinoma patient. These Kaplan–Meier curves in Figure 1, *A* and *B*, are a subset of the data presented in Supplemental Figure S1. A) Kaplan–Meier survival curves of invasive ductal breast carcinoma patients with high OSM and VEGF expression (upper quartiles, respectively) present diminished survival when compared to individuals with low OSM and VEGF expression (lower quartiles, respectively). Comparable trends appear upon examination of the upper and lower quartiles of patients with B) OSMRβ and VEGF expression, C) IL-6 and VEGF expression, and D) IL-6Rα and VEGF expression, indicating significant differences between upper and lower quartile survival for each group. Survival of patients with invasive ductal breast carcinoma by E) LIF and VEGF expression and F) LIFRβ and VEGF expression are not significantly different. Analysis obtained from Oncomine dataset entitled Curtis Breast. Log-rank test *$P < .05$, **$P < .01$, ***$P < .001$.

pSTAT3 ELISA

Intracellular pSTAT3 levels were assessed by ELISA, in accordance with the manufacturer's protocol (Cat#7146, Cell Signaling). 50,000 cells were adhered to 24-well plates overnight in serum-free media. Cells were incubated for the indicated times, and cell lysates were collected using 1x Cell Lysis Buffer (Cat# 9803, Cell Signaling). The lysates were diluted 1:3 with blocking buffer (PBS-0.05% Tween 20, 1% IgG-free BSA) and assessed by ELISA.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 software. To compare multiple groups, one- or two-way analysis of variance were run with Tukey's and Bonferroni's post-test, respectively where appropriate on ELISA data. Experiments were considered statistically significant if p values were less than 0.05. Error bars represent mean ± S.E. Experiments were performed at least three times to determine statistical significance.

Results

Inflammatory Cytokine and VEGF Co-Expression is Correlated with Decreased Survival of IDC Patients

To address the clinical importance of IL-6 family cytokines in invasive ductal carcinoma (IDC), we correlated patient survival relative to expression levels of VEGF with OSM, IL-6, LIF, or their specific receptor subunits. Using the Curtis METABRIC Breast dataset (47) obtained from Oncomine [48], co-expression in IDC patients was compared to survival and quantified by individual quartiles, as demonstrated for OSM and VEGF (Figure S1*A*) and OSMRβ and VEGF (Figure S1*B*). Utilizing just the upper and lower quartiles, we observed a significant correlation between high co-expression of OSM and VEGF and decreased survival, relative to low expression of both OSM and VEGF (P = .0190, Figure 1*A*). Similarly, decreased survival was observed in patients who had high expression levels of OSMRβ and VEGF (P = .0012, Figure 1*B*), IL-6 and VEGF (P = .0005, Figure 1*C*), or IL-6Rα and VEGF (P = .0016, Figure 1*D*) relative to those with low co-expression of each respective gene pair. Patient survival was not statistically affected by co-expression levels of LIF and VEGF (P = .0578, Figure 1*E*) or LIFRβ and VEGF (P = .1020, Figure 1*F*). Together, these results highlight the importance of tumor cell co-expression of VEGF with OSM, OSMRβ, IL-6, or IL-6Rα on the poor survival of individuals with IDC.

HER2- Status Dictates Poor Survival in IDC Patients with High Co-Expression of OSM, IL-6, OSMRβ or IL-6Rα and VEGF

As published studies have demonstrated the importance of receptor status in breast cancer recurrence and treatment [48–50], we next analyzed HER2 status on the survival of IDC patients according to VEGF and IL-6 family molecule co-expression. A statistically significant decrease in survival was only observed in HER2- patients with high OSM and VEGF expression (P = .0016) and not in HER2+ patients

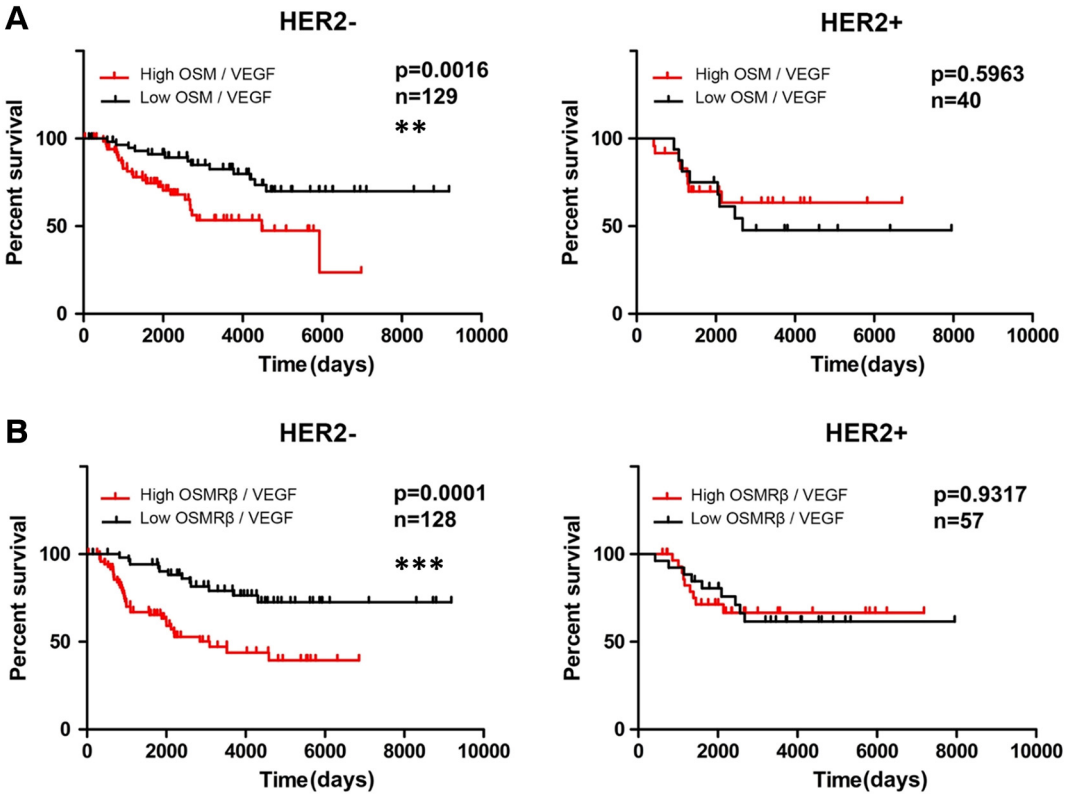

Figure 2. Co-expression of VEGF with OSM or OSMRβ appears to affect survival in HER2- but not HER2+ subtypes. A) Kaplan–Meier survival curves of invasive ductal breast carcinoma for HER2- patients and HER2+ patients by OSM and VEGF expression. In HER2- patients, high OSM and high VEGF expression (upper quartiles, respectively) is strongly correlated with decreased survival when compared to patients with low OSM and low VEGF expression (lower quartiles, respectively). However, this trend is not evident in HER2+ individuals, as no significant difference is observed between survival of patients with OSM and VEGF expression in upper and lower quartiles, respectively. Similar trends are observed in Kaplan–Meier survival curves for B) HER2- and HER2+ by OSMRβ and VEGF expression. Analysis obtained from Oncomine dataset entitled Curtis Breast. Log-rank test *P < .05, **P < .01, ***P < .001.

(P = .5963, Figure 2*A*). Decreased survival was also seen in HER2- individuals with high OSMRβ and VEGF co-expression (P = .0001, Figure 2*B*), high IL-6 and VEGF co-expression (P = .0005, Figure S2*A*), and high IL-6Rα and VEGF co-expression (P = .0112; Figure S2*B*). No statistically significant change in survival was detected with the respective HER2+ individuals (High OSMRβ/VEGF, P = .9317, Figure 2*B*) (High IL-6/VEGF, P = .6106, Figure S2*A*) (High IL-6Rα/VEGF, P = .0671, Figure S2*B*). High expression of LIF with high expression of VEGF also was correlated with poor survival in HER2- (P < .0001, Figure S2*C*), but not HER2+ IDC patients (P = .2806 Figure S2*C*). However, no association between HER2 status and survival was observed in IDC patients expressing high levels of LIFRβ and VEGF (P = .1247 and P = .534, respectively, Figure S2*D*). The effect of estrogen receptor (ER) status on patient survival was also investigated.

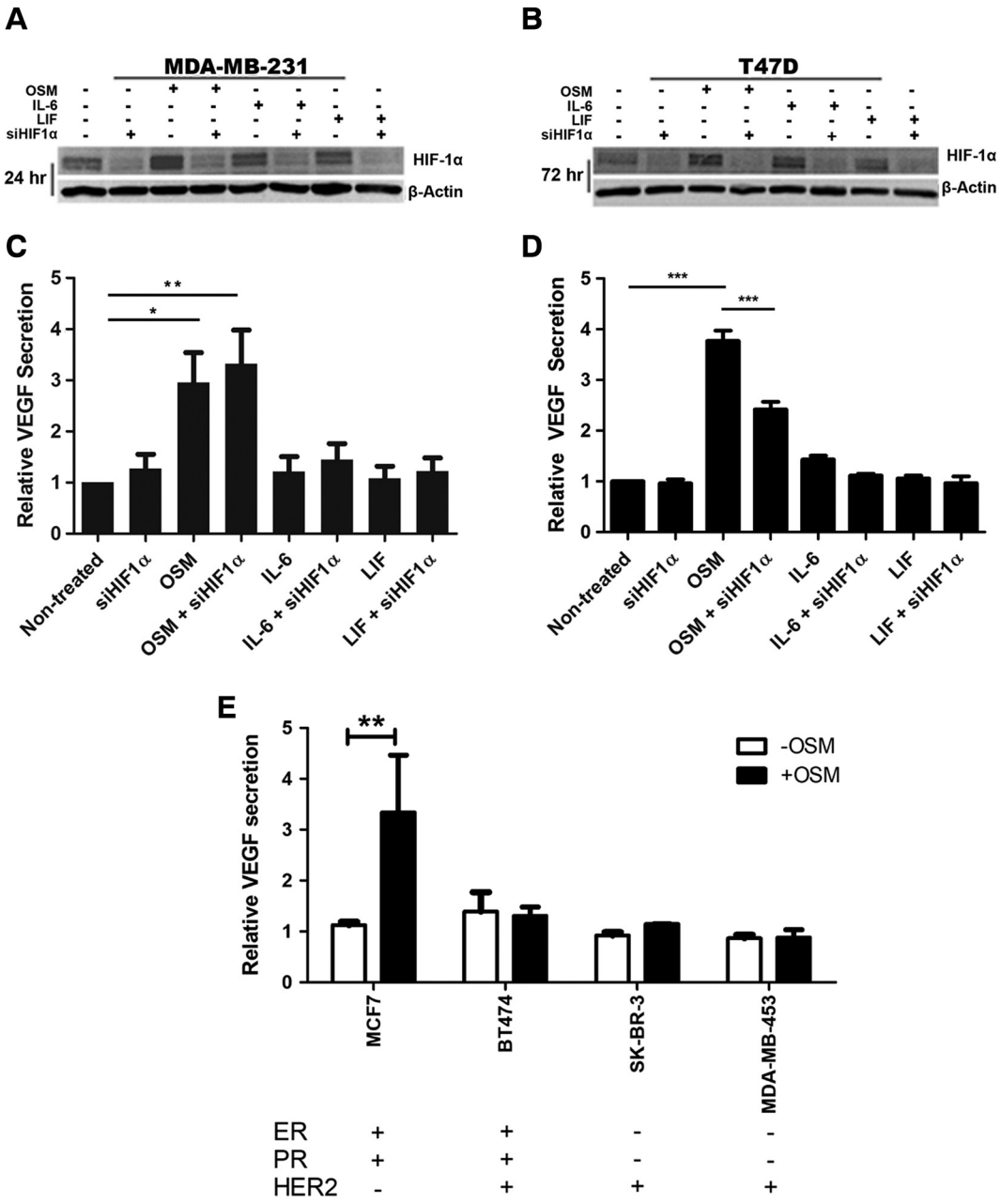

Figure 3. IL-6 family cytokine OSM induces VEGF secretion independent of HIF1α signaling in MDA-MB-231 cells. A) Treatment with OSM, IL-6, and LIF (25 ng/mL) for 24 hours promotes expression of HIF1α in MDA-MB-231 TNBC cells. Addition of an siRNA targeting HIF1α (siHIF1α) ablates the effect of adding cytokines. B) T47D cells treated with IL-6 family cytokines for 72 hours express greater levels of HIF1α relative to non-treated control. Treatment with siHIF1α reduces this effect, as assessed by immunoblot analysis. Immunoblots are representative of at least 3 experiments. Induction of VEGF secretion is observed following treatment with IL-6 family cytokines (25 ng/mL) for 24 hours in C) MDA-MB-231 cells (n = 5) and for 72 hours in D) T47D cells (n = 3), as determined by ELISA. Treatment with siHIF1α does not affect VEGF secretion in OSM-treated MDA-MB-231 cells; however, VEGF secretion is modestly reduced in T47D cells treated with OSM and siHIF1α. E) A panel of additional human breast cancer cell lines were tested for VEGF expression by ELISA. HER2- MCF7 cells have significant OSM-induced VEGF secretion, while HER2+ BT474, SK-BR-3, and MDA-MB-453 cells do not produce increased VEGF in response to OSM. One-way ANOVA, Tukey's post-test, $^{**}P$ < .01, $^{***}P$ < .001.

In HER2- IDC patients, high co-expression of OSM and VEGF in ER+ patients was associated with poor survival (P = .0008), while ER- status had no effect on survival (P = .6998, Figure S3*A*, B). ER status played a less significant role in patient survival when HER2 status was associated high OSMRβ and VEGF co-expression (Figure S3, *C* and *D*). These data suggest that while ER+ HER2- patients typically have fair prognoses [51], high tumor co-expression of OSM and VEGF negatively impacts survival in these individuals.

OSM Promotes VEGF Secretion from HER2- Breast Cancer Cells in a HIF1α Differential Manner

Our data suggested that, high OSM co-expression with the proangiogenic factor VEGF resulted in poor HER2- patient survival. To study VEGF expression and its transcription factor, HIF1α, in relation to IL-6 family cytokines, we used two distinct HER2- cell lines with differing ER status, the ER-negative MDA-MB-231 (ER- PR- HER2-) TNBC cell line and the ER-positive T47D (ER+ PR+ HER2-) cell line.

Treatment with OSM, IL-6, or LIF (25 ng/mL) induced HIF1α expression by western blot analysis in MDA-MB-231 cells at 24 hours and in T47D cells at 72 hours, relative to non-treated cells (Figure 3, *A* and *B*). These time points were selected as they were determined to have maximal HIF1α-induction in each cell line (data not shown). Despite the induction of HIF1α, treatment with IL-6 or LIF did not produce a significant change in the level of VEGF secreted (Figure 3, *C* and *D*). On the other hand, OSM treatment accounted for a 3-fold increase in VEGF secretion by ELISA in MDA-MB-231 cells at 24 hours ($P < .05$) and an almost 4-fold increase in T47D cells at 72 hours ($P < .001$) (Figure 3, *C* and *D*). Relative VEGF secretion was not altered in MDA-MB-231 cells treated with siHIF1α and OSM, while this same treatment reduced VEGF secretion by nearly 50% in T47D cells, demonstrating different signaling mechanisms. To confirm the importance of HER2- status on OSM-induced VEGF secretion, additional human breast cancer cells were investigated. A 3.3-fold increase in VEGF secretion was seen with ER+ HER2- MCF7 cells after a 72-hour treatment with OSM, as compared to untreated controls (Figure 3*E*). Importantly, in HER2+ BT474 (ER+), SK-BR-3 (ER-), and MDA-MB-453 (ER-) cells [20,52], OSM did not induce VEGF secretion (Figure 3*E*), despite the presence of OSMRβ in all cell lines (Figure S4*A*). Together, this data suggests that OSM-induced VEGF induction is likely mediated through different pathways in ER+ *versus* ER- HER2- cells.

To confirm a functional effect for OSM-induced VEGF production, we performed *in vitro* and *in vivo* angiogenesis assays. Conditioned media (CM) from MDA-MB-231 cells treated with OSM induced endothelial cell tube and branch point formation in human umbilical vein endothelial cells (Figure S5, *A* and *B*). Similarly, CM from OSM-treated MDA-MB-231 cells had a 12-fold increase in angiogenesis, as compared to CM from untreated MDA-MB-231 cells when mixed with Matrigel and inserted into athymic nude mice ($P < .001$, Figure S5, *C* and *D*). Together, these results suggest that OSM promotes the secretion of functional VEGF from breast cancer cells.

OSM Strongly Induces STAT3 Activation in ER+ and ER- HER2- Breast Cancer Cells

OSM is capable of binding to and activating both the OSMR and the LIFR, while LIF binds to only the LIFR and IL-6 binds to the IL-6 receptor. Stimulation of these receptors activate various signaling pathways including the STAT3 and JNK pathways [53–56]. First, we

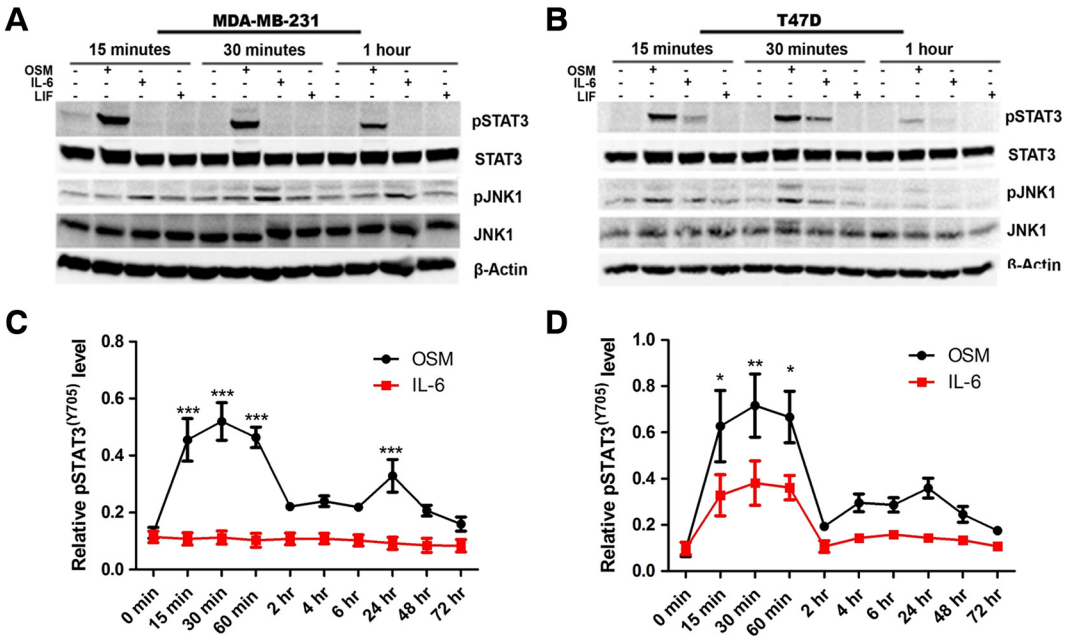

Figure 4. OSM strongly induces phosphorylation of STAT3. A) Treatment with OSM (25 ng/mL) for 15 minutes, 30 minutes, and 1 hour strongly induces phosphorylation of STAT3 (Tyr 705) in MDA-MB-231 cells and moderately induces JNK phosphorylation (T183/Y185). Treatment with either IL-6 or LIF (25 ng/mL) does not induce pSTAT3 or pJNK. B) Treatment with OSM induces phosphorylation of STAT3 and JNK in T47D cells. IL-6 induces moderate phosphorylation of STAT3 and JNK at early time points. Phosphorylation of STAT3 and JNK was compared to control β-actin, as assessed by immunoblot analysis. Blots are representative of three experiments. Time course experiment for STAT3 phosphorylation upon treatment with OSM or IL-6 (25 ng/mL) for 0.25–72 hours in C) MDA-MB-231 cells, and in D) T47D cells. Treatment with IL-6 does not induce pSTAT3 expression in MDA-MB-231 cells, while inducing moderate pSTAT3 expression in T47D cells. ELISA was performed in quadruplicate; two-way ANOVA with Bonferroni post-test, *$P < .05$, **$P < .01$, ***$P < .001$.

confirmed that these receptor elements (OSMRβ, LIFRβ, IL-6Rα, and gp130) were expressed in MDA-MB-231 and T47D cells by RT-PCR (Figure S4*B*). Next, we sought to determine the mechanism by which OSM induces the expression of VEGF by investigating the activation of the transcription factors STAT3 and JNK, found downstream of OSMR. Treatment with OSM (25 ng/mL) strongly induced phosphorylation of STAT3 (pSTAT3) in MDA-MB-231 TNBC cells at both 15 and 30 minutes, as assessed by immunoblot. In contrast, addition of OSM only slightly induced phosphorylation of JNK1 (pJNK1) in these cells (Figure 4*A*). Neither the addition of IL-6 nor LIF led to STAT3 or JNK1 phosphorylation in ER- MDA-MB-231 cells. In T47D cells, stimulation with either OSM or IL-6 promoted phosphorylation of STAT3 at 15 and 30 minutes (Figure 4*B*). Phosphorylation of STAT3 was not induced in ER+ T47D cells treated with LIF. A slight induction of JNK1 phosphorylation was observed following treatment with OSM in T47D cells, whereas IL-6 and LIF did not produce an effect. In both cell lines, levels of OSM-induced STAT3 phosphorylation were transient yet sustained over time up to 72 hours, with preferential activation around 0.5 hours, relative to treatment with IL-6 ($P < .001$, Figure 4, *C* and *D*). Interestingly, treatment with IL-6 has no observed effect on pSTAT3 levels in MDA-MB-231 cells and only induced moderate STAT3 phosphorylation in T47D cells.

Activation of STAT3 Signaling is Required for VEGF Secretion by MDA-MB-231 TNBC Cells

Our data suggest that OSM-induced VEGF is independent of HIF1α in MDA-MB-231 TNBC cells. To determine whether OSM promotes VEGF secretion *via* the STAT3 pathway, we performed VEGF ELISAs using siRNAs targeting STAT3 (siSTAT3) and JNK1 and 2 (siJNK1 and siJNK2). In MDA-MB-231 cells, treatment with OSM and siSTAT3 suppressed VEGF secretion (Figure 5*A*). However, this complete suppression was not observed in T47D cells, likely due to the role of HIF1α in the secretion of VEGF in this cell line (Figure 5*B*). While initial studies using a chemical inhibitor of JNK suggested that JNK signaling was in part necessary for OSM-mediated induction of VEGF (Figure S6*A*), further investigations revealed that these effects might have resulted from the off-target suppression of STAT3 phosphorylation (Figure S6*B*). However, siRNAs targeting both JNK1 and JNK2 (siJNK1 and siJNK2, respectively) had no effect on the levels of VEGF secretion in MDA-MB-231 or T47D cells treated with OSM (Figure 5, *C* and *D*). Together, these results demonstrate that OSM-mediated induction of VEGF is dependent on STAT3 in MDA-MB-231 cells, while T47D cells utilize STAT3, HIF1α, and possibly other pathways to promote VEGF production in response to OSM.

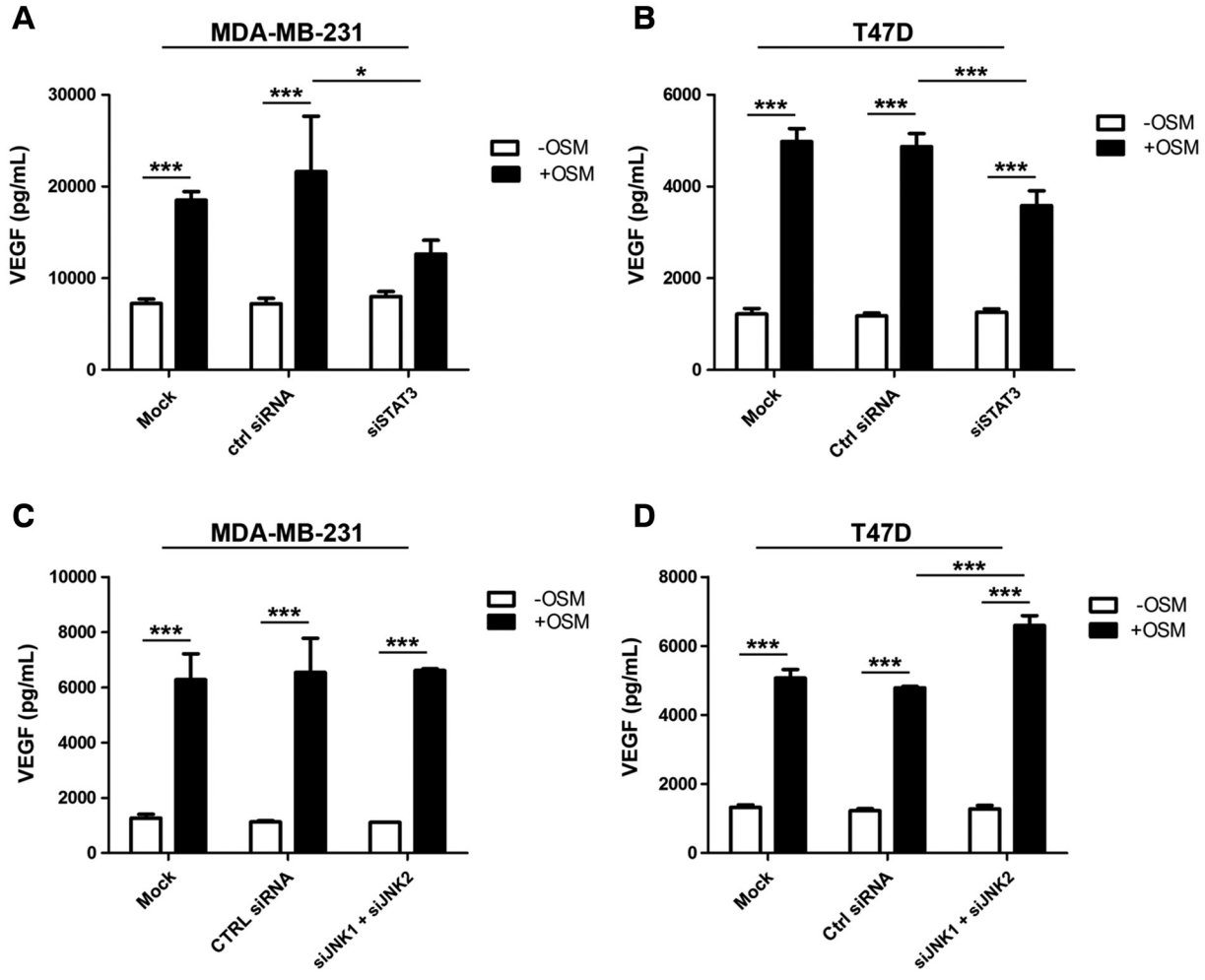

Figure 5. OSM induces VEGF *via* STAT3 signaling. A) Treatment with OSM (25 ng/mL) and siSTAT3 suppresses VEGF secretion by 3-fold in MDA-MB-231 cells. B) Treatment with OSM and siSTAT3 moderately reduces VEGF secretion by T47D cells. VEGF secretion was not reduced by the addition of siRNAs targeting both JNK1 and JNK2 in either C) MDA-MB-231 or D) T47D cells. Experiments were performed in triplicate two-way ANOVA with Bonferroni post-test, *$P < .05$, ***$P < .001$.

Discussion

The different subtype-specific molecular characteristics of human breast cancer have important implications for clinical treatment options, disease progression, and ultimately patient prognosis. In this study, we show the correlation of high tumor cell co-expression of VEGF with inflammatory cytokines (OSM and IL-6), or their respective receptors (OSMRβ and IL-6Rα), with the poor survival of individuals with HER2- invasive ductal carcinoma. This may be caused by the fact that OSM, IL-6, and their receptors are associated with increased metastatic capacity [57,58], including increased invasion and migration [20,24,25,29,59] and the promotion of angiogenesis [26,27,31,60]. Moreover, OSM has been shown to bind to extracellular matrix proteins such as collagens, laminins, and fibronectins in an active conformation; thereby, accumulating in a breast tumor microenvironment to further promote these effects [60,61]. Subsequently, we demonstrate that OSM induces VEGF in HER2-, but not HER2+, breast cancer cells and that ER+ and ER- HER2- tumor cells respond differently to IL-6 cytokines in their induction of VEGF. Together, our findings suggest that VEGF, OSM, and IL-6 or their receptors can be used as potential negative prognostic markers and therapeutic targets for breast cancer.

Our clinical analysis for this work included 1245 IDC patients subdivided by HER2 and ER status. When IDC patients were subdivided by HER2 status, a marked decrease in overall survival was found in HER2- patients exhibiting high OSM or OSMRβ and VEGF co-expression compared to HER2+ patients. HER2- patients also fared much worse when their tumors had high co-expression of IL-6 or IL-6Rα and VEGF. This data demonstrate clear statistical significance for breast cancer patients with HER2-, but not HER2+ status. This was further supported by our *in vitro* analyses showing that untreated HER2+ BT474 breast cancer cells constitutively express a basal level of VEGF [62] and that OSM had no additional effect on the induction of VEGF secretion in HER2+ cells such as BT474, SK-BR-3, and MDA-MB-453. These findings may suggest redundancy between HER2 and inflammatory cytokine-induced signaling, with most IDC tumors requiring overactivation of only one or the other pathway. Other studies show that,

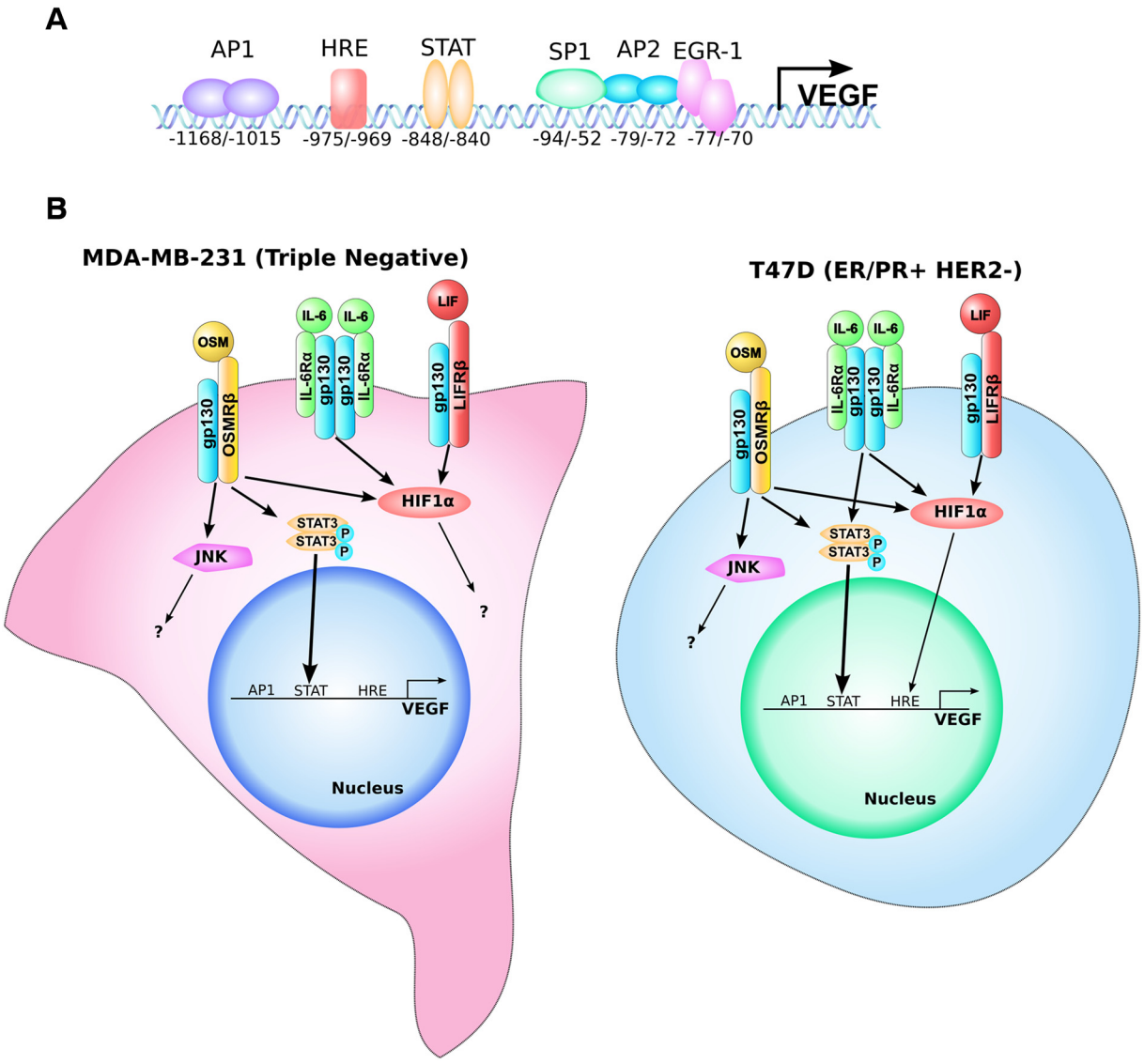

Figure 6. Mechanistically distinct regulation of VEGF secretion in MDA-MB-231 TNBC and T47D (ER+/PR+/HER2-) cells. A) The VEGF promoter can be activated by various transcription factors. B) In MDA-MB-231 cells, OSM mediates VEGF secretion by activating the STAT3 signaling pathway downstream of the OSMR (OSMRβ + gp130). In T47D cells, OSM regulates VEGF secretion *via* both HIF1α and the STAT3 signaling pathway.

IL-6 family cytokines have been proposed as profiling markers for HER2- breast cancer, specifically TNBC, while having no significant correlation with HER2+ breast cancer [37]. Overall, these results indicate that the inflammatory cytokines OSM and IL-6 contribute to the aggressive phenotype seen with HER2- disease.

When investigating clinical IDC patient data with respect to ER status, we were able to observe a significant negative effect on survival in ER+/HER2- individuals with high co-expression of VEGF and OSM or OSMRβ. This is interesting in light of the fact that OSM has been shown to negatively regulate expression of the estrogen receptor itself [61]. Such regulation may indicate a key element in OSM-driven malignancy, with the possibility that ER+ tumor cells lose ER status over time and evolve to become less susceptible to hormone therapies and more difficult to treat [61]. Furthermore, several *in vitro* studies have demonstrated that OSM has a greater effect inducing epithelial-mesenchymal transition (EMT) and tumor cell detachment effects in ER+ PR+ HER2- breast cancer cell lines, such as T47D and MCF7, than on ER- TNBCs like MDA-MB-231 or MDA-MB-468 ([27,63,64]; data not shown).

Standing alone, these results suggest important differences between breast cancer subtypes in relation to VEGF and IL-6 family inflammatory cytokines. As our patient data suggest that high coexpression of IL-6 family cytokines and VEGF levels leads to poor survival in HER2- breast cancer, we investigated two HER2- breast cancer cell lines: ER- MDA-MB-231 and ER+ T47D. In normal physiological conditions, VEGF expression is regulated under both hypoxic and non-hypoxic conditions, as the promoter region contains several response elements, including an hypoxia response element (HRE) for HIF1, that allow for VEGF regulation downstream of various signaling pathways (Figure 6*A*, [38–41]). Our studies showed that OSM, IL-6, and LIF each increased expression of HIF1α, an important transcription factor for VEGF, but only OSM had a significant effect on VEGF production levels. Additionally, there were key differences in the signaling pathways inducing VEGF secretion. Specifically, we showed that T47D cells utilize STAT3, HIF1α, and possibly other pathways, while MDA-MB-231 cells relied solely on STAT3 activation for OSM-induced VEGF secretion. (Figure 6*B*). In T47D cells, it is possible that STAT3 and HIF1α directly interact with each other to induce VEGF. Pawlus et al. demonstrated that STAT3 and HIF1α immunoprecipitate together in breast cancer cells and may have synergistic effects when both pathways are activated [41]. Additionally, suppression of HIF1 signaling by HIF1α siRNA did not decrease hypoxia-induced STAT3 phosphorylation, which suggests that STAT3 activation is not dependent on HIF1 signaling [65]. Together, these results may indicate unique properties pertaining to angiogenic signaling in ER- TNBC *versus* ER+ PR+ HER2- tumors.

Interestingly, although it might be expected that IL-6 should also induce VEGF secretion from breast cancer cells, we found that in ER- MDA-MB-231 cells, IL-6 did not activate the STAT3 pathway and therefore did not promote VEGF secretion. Previous studies have shown that IL-6 has no effect on some markers of metastatic fitness such as E-cadherin levels in MDA-MB-231 cells, which are signaled through the JAK/STAT3 pathway [66]. This suggests that while ER- MDA-MB-231 cells express the IL-6 receptor, STAT3 signaling is not necessarily regulated by IL-6 in these cells. While we showed that IL-6 weakly activated the STAT3 pathway in ER+ T47D cells, this induction was only seen in the first hour of treatment and did not extend to 48 hours as did OSM-induced pSTAT3. Overall, the activation of STAT3 signaling by OSM is significantly greater than with IL-6 and highlights a difference in signaling magnitude and possibly a key functional distinction between these two cytokines in a breast cancer subtype-specific manner.

Taken together, our studies demonstrate that co-expression of VEGF and IL-6 family cytokine molecules emerge as potential negative prognostic markers particularly for HER2-, but not HER2+, invasive ductal breast carcinoma. Our follow-up *in vitro* experiments demonstrate distinct differential cytokine-induced STAT3 and HIF1α signaling, which lead to varied levels of VEGF secretion among various TNBC and ER+ PR+ HER2- breast tumor cells. These breast cancer subtype-specific differences in HIF1α signaling could complicate anti-cancer therapies targeting HIF1, which are currently undergoing clinical trials [67]. Collectively, these results suggest that therapeutic inhibition of IL-6-family cytokines such as OSM may lead to VEGF suppression and improved patient survival in HER2- disease.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.10.004.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Author's Contributions

All authors contributed substantially to the concept of the paper and work presented in this study. Ken Tawara designed and ran the experiments for Figures 4, *D* and *E*, and 5, and Figure S5, interpreted the data, constructed Figure 6, and helped write and made substantial edits to the manuscript. Hannah Scott and Jacqueline Emathinger helped collect and interpret data from Oncomine for Figures 1 and 2 and Figure S1-S3, and also helped write the manuscript with help from Danielle Hedeen and Madhuri Nandakumar. They also helped with the conceptual design for Figure 6. Alex Ide contributed to data in Figure S5, and Andrew Older contributed to data in Figure S5. Ryan Fox contributed to data for Figures 3 and 4, *B* and *C*, Daniel Greiner contributed to data in Figure 3 and Figure S4*A*, and Dollie LaJoie contributed to the data for Figure S4*B*. Ryan Holzer contributed initial conceptual guidance and preliminary data for the overall concept of the paper. Cheryl Jorcyk, who as the principal investigator, provided conceptual guidance for all aspects of the project.

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