An Autocrine VEGF/VEGFR2 and p38 Signaling Loop Confers Resistance to 4-Hydroxytamoxifen in MCF-7 Breast Cancer Cells

Reidun Aesoy,[1] Betzabe Chavez Sanchez,[1] Jens Henrik Norum,[2] Rolf Lewensohn,[1] Kristina Viktorsson,[1] and Barbro Linderholm[1,3]

[1]Department of Oncology/Pathology, Karolinska Biomics Center, Karolinska Institutet, Stockholm, Sweden; [2]Department of Pharmacology, University of Oslo, Oslo, Norway; and [3]Department of Oncology, Linkoping University Hospital, Linköping, Sweden

Abstract

Tamoxifen, a partial estrogen receptor antagonist, is part of the standard treatment of both primary and advanced breast cancers. However, significant proportions of breast cancers are either *de novo* resistant or develop tamoxifen resistance during the course of treatment through mechanisms which have been only partly characterized. We have previously found that high vascular endothelial growth factor (VEGF) or VEGF receptor 2 (VEGFR2) expression and concomitant high p38 mitogen-activated protein kinase activity within breast cancers predict a poor outcome for tamoxifen-treated patients. Here, we have molecularly dissected how VEGF/VEGFR2 and p38 are linked, and contribute to tamoxifen resistance within breast cancer using a MCF-7 BC cell model with different 4-hydroxytamoxifen (4-OHT) responsiveness. We report that MCF-7 breast cancer cell lines with tamoxifen resistance have increased secretion of VEGF and increased signaling through VEGFR2 compared with parental MCF-7 cells. 4-OHT treatment caused the ablation of VEGF secretion in parental MCF-7 cells, whereas in the tamoxifen-resistant subline, a VEGF/ VEGFR2 signaling loop was still evident upon treatment. Increased basal levels of total and phosphorylated p38 were observed in tamoxifen-resistant cells. Pharmacologic inhibition of p38 reduced the proliferation of both tamoxifen-responsive and tamoxifen-resistant cells and showed an additive growth-inhibitory effect in combination with 4-OHT. A connection between VEGF/ VEGFR2 and p38 signaling was identified by VEGF and VEGFR2 knockdown, which equally reduced both the total and the active forms of p38 in tamoxifen-resistant cells. Taken together, our results suggest that decreased sensitivity to 4-OHT is caused by a death-protecting VEGF/VEGFR2 and p38 growth factor loop in breast cancer cells. Inhibition of these signaling pathways may be beneficial to overcome tamoxifen resistance. (Mol Cancer Res 2008;6(10):1630–8)

Introduction

Approximately 75% of all patients with breast cancer have a tumor expressing the estrogen receptor (ER) and/or the progesterone receptor. These tumors depend on estrogens for their survival and endocrine therapy is used to deprive breast cancer cells of such stimuli either through competitive binding to ER or by estrogen deprivation (1). The antiestrogen tamoxifen, a nonsteroidal triphenylethylene derivate, has been used extensively in the clinical management of primary and advanced endocrine-sensitive breast cancer. Tamoxifen exerts its antiproliferative effect by binding competitively to ER, thereby blocking the mitogenic effect of estrogens. Although beneficial for a significant proportion of breast cancers, some patients display *de novo* or acquired resistance to tamoxifen through mechanisms which are not yet fully understood.

In tumors, enhanced growth factor signaling has been found to increase proliferation, cell survival, migration, invasiveness, and angiogenesis, resulting in a more aggressive tumor phenotype (2). Upon growth factor receptor activation, downstream intracellular signaling cascades such as the mitogen-activated protein kinase pathway, i.e., extracellular signal-regulated kinase (ERK or p42/44^{MAPK}), c-*Jun* NH$_2$-terminal kinase (JNK)/stress-activated protein kinase, and p38 become activated. ERK mainly regulates cell survival and differentiation, whereas p38 and JNK may promote cell survival in some cell systems; however, in other cells, they induce cell death (3). Increased ERK, p38, and phosphoinositide-3-kinase/ Akt signaling, as a consequence of either epidermal growth factor receptor or human epidermal growth factor receptor 2 (HER2) signaling within the tumor, have been shown to block the antiproliferative effect of tamoxifen (4-7).

Angiogenesis, a hallmark of tumorigenesis, is necessary to provide the tumor with oxygen and nutrients (8). Several different growth factors and cytokines drive angiogenesis with

VEGF as the predominant proangiogenic factor in human cancer (9). Furthermore, VEGF production in tumors or in endothelial cells may stimulate cell proliferation, migration, and invasion, which is mainly mediated via VEGF receptor 2 (VEGFR2/KDR) signaling (10). Upon ligand interaction, VEGFR2 is activated through receptor dimerization and autophosphorylation of tyrosine residues, e.g., Y951, Y1175, and Y1214 in its cytoplasmic kinase domain (9). In addition to hypoxia, estrogen and progesterone signaling have been shown to increase VEGF expression via a putative estrogen-responsive element within the VEGF promoter (11). The human VEGF-A gene contains eight exons and may be alternatively spliced into at least four different transcripts (9). Although the $VEGF_{189}$ and $VEGF_{206}$ isoforms bind to heparin-containing proteoglycans in the extracellular matrix, $VEGF_{121}$ is an acidic polypeptide that fails to bind heparin and is a freely diffusible protein. The predominant isoform $VEGF_{165}$ is secreted, however, a significant fraction remains bound to the cell surface and extracellular matrix.

As described above, increased HER2 signaling is implicated in tamoxifen resistance. A connection between HER2 amplification and increased VEGF expression involving both the phosphoinositide-3-kinase/AKT as well as ERK signaling pathways has been reported in breast cancer (12, 13). VEGF expression may contribute to the aggressive phenotype seen in HER2-positive breast cancer, however, VEGF is also expressed in a considerable number of HER2-negative tumors suggesting that VEGF expression is regulated by additional processes in breast cancer (14, 15).

We and others have previously reported that high expression of VEGF or VEGFR2 within breast cancer is associated with shorter survival for breast cancer patients when placed on adjuvant tamoxifen treatment (16-19). However, patients without detectable levels of activated p38 had a good prognosis following adjuvant tamoxifen treatment despite high VEGF or VEGFR2 levels, suggesting VEGF/VEGFR2 and p38 signaling to be of importance in tamoxifen resistance.[4] The aim of this study was to characterize such networks in breast cancer cell lines. We show that in MCF-7 breast cancer cells, loss of response to tamoxifen treatment is, at least in part, caused by an active autocrine VEGF/VEGFR2/p38 signaling loop.

Results

Viability of MCF-7 and LCC2 Cells on 4-Hydroxytamoxifen Treatment

In order to investigate a possible connection between VEGF/VEGFR2 and p38 and their role in development and maintenance of 4-hydroxytamoxifen (4-OHT) resistance in breast cancer cells, we adopted a previously characterized 4-OHT resistance breast cancer model consisting of the parental MCF-7 cells and its 4-OHT–resistant counterpart, MCF-7/LCC2 (20). The latter is derived from MCF-7 by sequentially
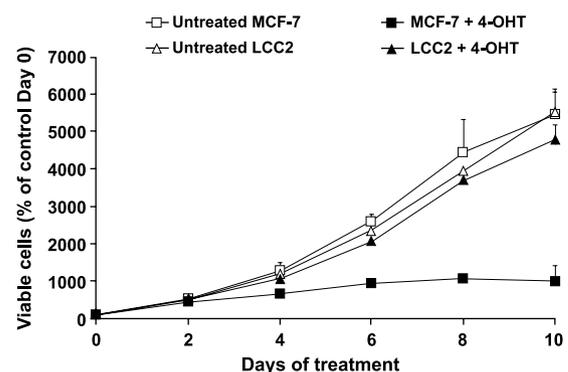
exposing MCF-7 cells to increasing concentrations of 4-OHT (20). In agreement with Brunner and colleagues, we found that the LCC2 cells showed significantly less decrease in viability upon 4-OHT treatment compared with parental MCF-7 cells using a trypan blue assay over a period of 10 days (Fig. 1). Decreased ERα expression is known to cause tamoxifen resistance, however, in line with previous reports using this cell model, as well as findings from clinical studies showing retained expression of functional ER in breast cancer patients with acquired resistance to tamoxifen, we found that both MCF-7 and LCC2 express ERα to a similar extent and its expression was not affected by 4-OHT treatment (Supplementary Fig. S1; refs. 20, 21).

Higher Secretion of VEGF Is Observed from Tamoxifen-Resistant Compared with Sensitive Breast Cancer Cells

Using an ELISA assay detecting both $VEGF_{165}$ and $VEGF_{121}$, the basal secretion of VEGF as well as VEGF secretion after 4-OHT treatment were compared between MCF-7 and the 4-OHT–resistant subline LCC2. The basal secretion of VEGF was found to be on average 4.8 ng/mL from 1×10^6 MCF-7 cells and 8.2 ng/mL from 1×10^6 LCC2 cells, i.e., 1.7 times more VEGF was secreted from LCC2 cells compared with the same amount of MCF-7 cells when examined 48 hours after seeding. VEGF secretion was then compared between the two cell types at day 6 after the addition of 4-OHT (Fig. 2), a time point when a significant difference in viability was found between MCF-7 and LCC2 cells (see Fig. 1). A major difference in VEGF secretion between the cell lines was seen following 4-OHT treatment. 4-OHT (0.1 μmol/L) was enough to suppress the secretion of VEGF from MCF-7 cells (Fig. 2). In contrast, secretion of VEGF from LCC2 cells was still evident, and clearly higher than for MCF-7 cells, at a high dose of 4-OHT (1 μmol/L; Fig. 2).

Increased VEGFR2 Signaling Is Observed in Tamoxifen-Resistant Breast Cancer Cells

Next, we examined if VEGFR2 expression and activation differed between MCF-7 and LCC2 cells. Basal and treatment-induced total and phosphorylated levels of VEGFR2 in MCF-7

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[4] Unpublished data by Linderholm B, Hellborg H, Johansson U, von Schoultz E, Hagerstrom T, Skoogh L. Co-expression of VEGF and VEGFR2, both factors significantly correlated to activated p38 mitogen protein kinase (MAPK) and worse outcome in oestrogen (ER)- and progesterone receptor (PgR) positive breast cancer patients receiving adjuvant Tamoxifen.

FIGURE 1. Characterization of 4-OHT responses in MCF-7 and LCC2 cells. MCF-7 and LCC2 cells were treated with 1 μmol/L of 4-OHT for 2, 4, 6, 8, and 10 days and cell viability was assessed using trypan blue assay. Points, mean of two independent experiments with duplicate samples in each experiment; bars, SD.
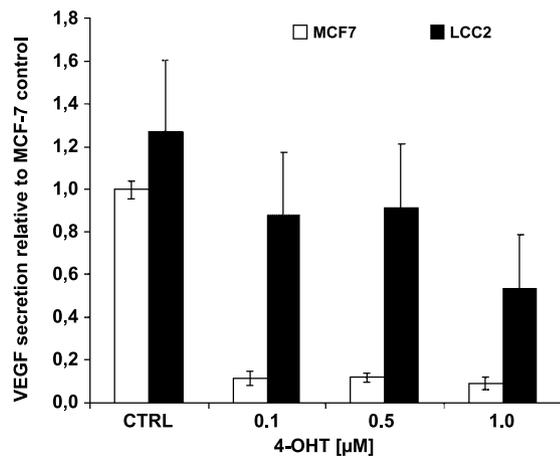
FIGURE 2. Secretion of VEGF from MCF-7 and LCC2 cells. MCF-7 and LCC2 cells were left untreated or treated with 4-OHT at the indicated doses for 6 days and the secretion of VEGF into the cell culture medium was determined by ELISA assay. The VEGF secretion is given relative to untreated (*CTRL*) MCF-7 cells; columns, mean of three experiments done in duplicate; bars, SD.

and LCC2 cells were examined by Western blot analysis (Fig. 3). Untreated MCF-7 and LCC2 cells were found to express VEGFR2 to a similar extent (Fig. 3A and B). However, a higher portion of VEGFR2 was found to be phosphorylated at Tyr[1175] in tamoxifen-resistant LCC2 cells than in sensitive MCF-7 cells (Fig. 3A and C). 4-OHT treatment caused a decrease of VEGFR2 in MCF-7 as well as, although to a lesser extent, of the phosphorylated form (Fig. 3). In contrast, in LCC2, the total amount of VEGFR2 was rather unaffected by 4-OHT treatment, whereas the degree of phosphorylation was decreased but still as high as in untreated MCF-7 cells after a dose of 2 μmol/L 4-OHT (Fig. 3).

Implication of the Mitogen-Activated Protein Kinase p38 in Tamoxifen-Resistant Breast Cancer Cells

When analyzing clinical material from patients with breast cancer, we found that breast cancer tumors with high VEGF/VEGFR2 and p38 levels were associated with less overall survival of the patients when placed on tamoxifen therapy.[4] Our hypothesis was, therefore, that in tamoxifen-resistant breast cancer cells, p38 is activated partly as a consequence of high VEGF/VEGFR2 signaling, creating a positive feedback loop thus enabling the cells to proliferate even in the presence of 4-OHT. In order to test this hypothesis, we examined the expression level of p38 as well as its degree of phosphorylation in MCF-7 and LCC2 cells in the presence and absence of 4-OHT treatment. In untreated cells, an increased expression of total p38 was observed in LCC2 compared with parental MCF-7 cells (Fig. 4A and B). After 6 days of 4-OHT treatment, the expression of total p38 was unchanged in MCF-7, although slightly increased in LCC2 cells (Fig. 4A and B). Interestingly, 4-OHT treatment caused opposite effects on the degree of p38 activity in the two cell lines. Although the phosphorylation of p38 increased in a dose-dependent manner in MCF-7, it decreased in LCC2 (Fig. 4A and B), an observation that is also seen when comparing the phosphorylated p38 to the total expression level (Fig. 4C). Furthermore, we examined the
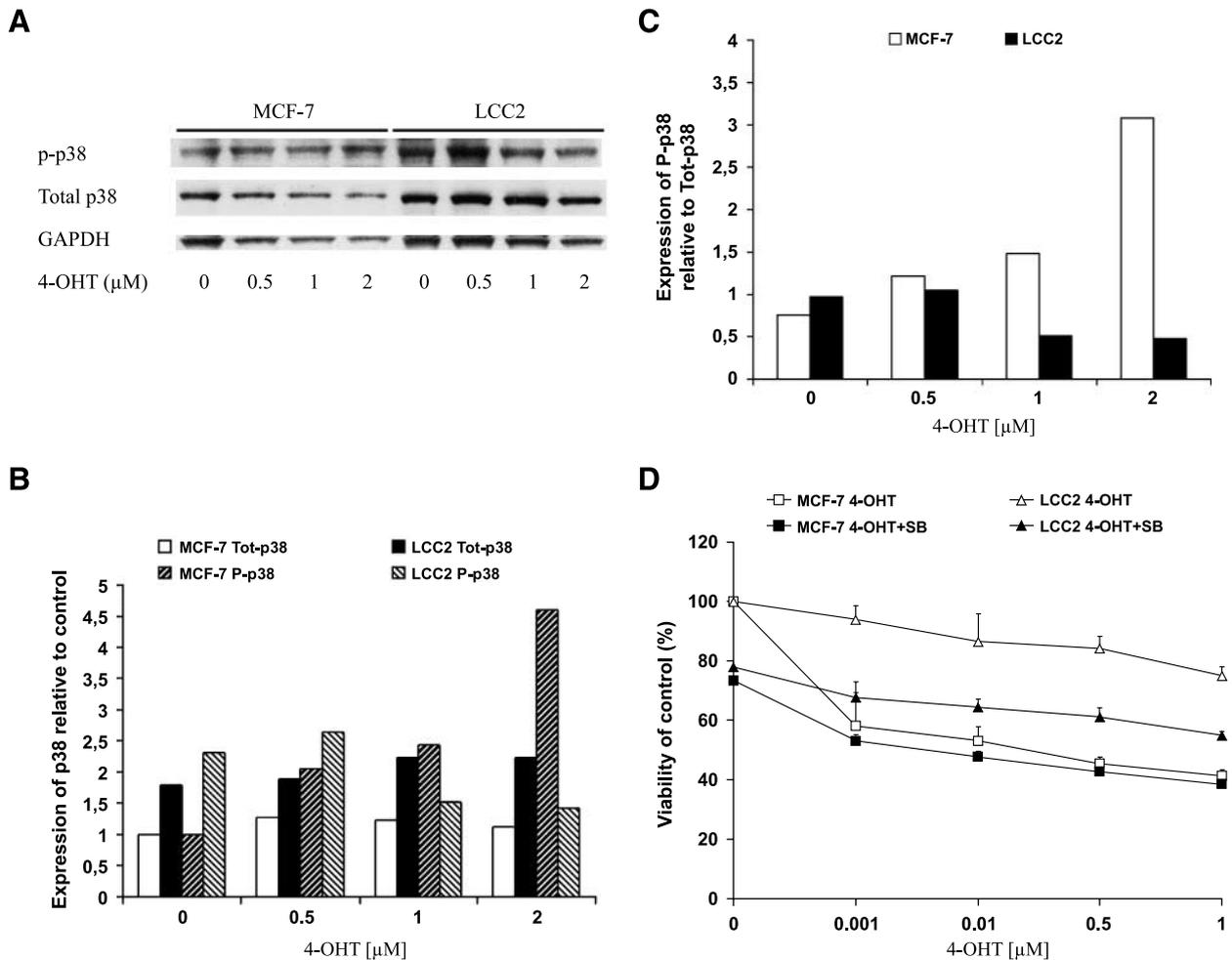
influence of p38 inhibition on cell proliferation. MCF-7 and LCC2 cells were treated with the competitive p38 inhibitor SB202190 alone or in combination with 4-OHT. Treatment with 10 μmol/L of SB202190 reduced the cell proliferation of both MCF-7 and LCC2 cells with ~20% (Fig. 4D). For the combination treatments, the cells were pretreated for 6 hours with 10 μmol/L of the p38 inhibitor SB202190 before the addition of the indicated concentrations of 4-OHT. Inhibition of p38 in combination with 4-OHT gave an additive effect on inhibition of both MCF-7 and LCC2 cell viability (Fig. 4D).

Implication of JNK in Tamoxifen-Resistant Breast Cancer Cells

Given that p38 and JNK often show redundant functions and are partially regulated by the same upstream signaling

A

B

C
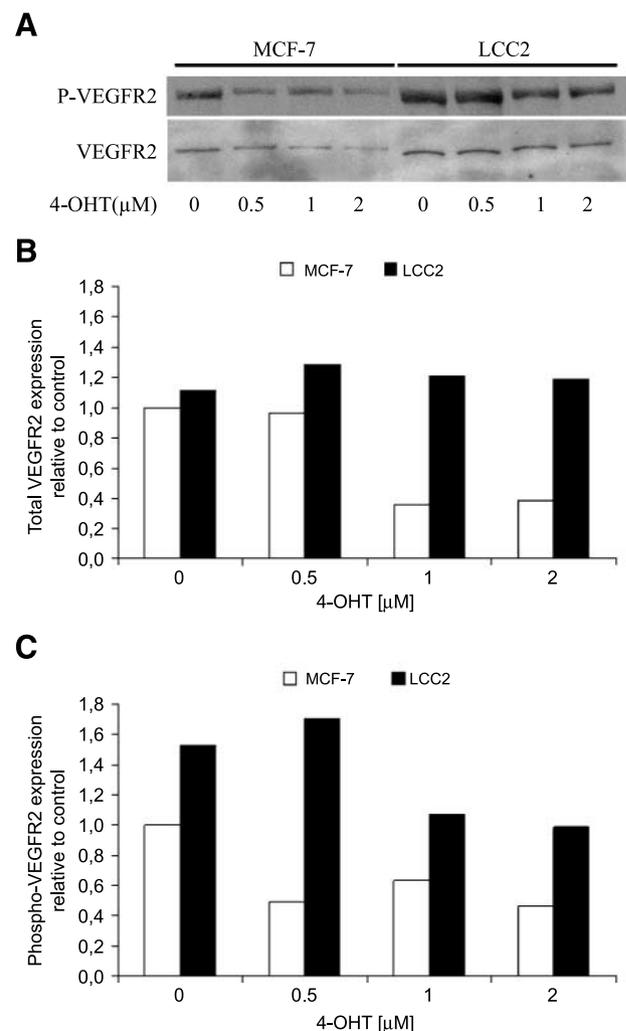
FIGURE 3. Expression of VEGFR2 in MCF-7 and LCC2 cells. **A.** MCF-7 and LCC2 cells were left untreated or treated with 4-OHT at the indicated doses for 6 days and the total expression and degree of phosphorylation of Tyr[1175]-VEGFR2 were determined by Western blot analysis. **B.** Quantified levels of total VEGFR2 expression in **A**. **C.** Quantified levels of phosphorylated VEGFR2 in **A**. The expressions are given as fold relative to respective expression in the untreated MCF-7 cell sample (**B** and **C**). Due to space limitations, the gel pictures have been cropped and full-length gels are presented in Supplementary Fig. S4.

FIGURE 4. Expression of p38 in MCF-7 and LCC2 cells and the influence of p38 inhibition on breast cancer cell survival. **A.** MCF-7 and LCC2 cells were left untreated or treated with 4-OHT at the indicated doses for 6 days and the basal and 4-OHT treatment–induced changes in total expression and degree of phosphorylation of p38 were analyzed by Western blot. **B.** Quantified levels of total and phosphorylated p38 from the Western blot in **A**. The expression and activation upon treatment are given as fold relative to the untreated MCF-7 cell sample after correcting for loading through glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. **C.** Quantified degree of phosphorylated p38 relative to total p38 expression in **A**. **D.** The effect of p38 inhibition on breast cancer cell survival and 4-OHT responses was examined by treating the cells with 4-OHT in the presence and absence of SB202190 (10 μmol/L, added 6 h prior to 4-OHT). Cell viability was examined 6 days after the addition of 4-OHT using the MTT assay. Viability is given as a percentage of untreated control. Points, mean of three independent experiments with triplicate samples. The gel pictures have been cropped and full-length gels are presented in Supplementary Fig. S5.

components, we also analyzed if an increased expression and activation of JNK occurred in tamoxifen-resistant breast cancer cells. Western blot analysis showed that LCC2 cells have an ∼ 2-fold higher expression of JNK2 than MCF-7 (Fig. 5A and C), whereas the expression of JNK1 was almost the same in the two cell lines (Fig. 5A and B). When comparing LCC2 with MCF-7 cells, the phosphorylated form of JNK1 was increased almost 5-fold (Fig. 5A and B) and JNK2 ∼ 2-fold (Fig. 5A and C). Thus, there is an increase in basal JNK activity in the tamoxifen-resistant LCC2 compared with the sensitive MCF-7 cells. 4-OHT treatment had no effect on total JNK1 expression in either cell type and relatively little effect on its phosphorylation in LCC2 cells. However, in MCF-7 cells, the phosphorylation of JNK1 increased dose-dependently (Fig. 5A and B). The JNK2 level in MCF-7 cells was somewhat increased upon 4-OHT treatment, whereas it decreased in LCC2 cells (Fig. 5A and C). In MCF-7 cells, the degree of JNK2

phosphorylation was induced by 1 μmol/L of 4-OHT, whereas it was down-regulated in LCC2 cells compared with its levels in the respective untreated cell type (Fig. 5A and C). In order to examine the effect of JNK inhibition on viability, we treated MCF-7 and LCC2 cells with the competitive JNK inhibitor SP600125, alone or in combination with 4-OHT. Treatment with 10 μmol/L of SP600125 reduced the proliferation of MCF-7 and LCC2 cells with ∼ 70% and 40%, respectively (Fig. 5D). For the combination treatments, the cells were pretreated for 6 hours with 10 μmol/L of the JNK inhibitor before the addition of the indicated concentrations of 4-OHT. In the viability assay, SP600125 in combination with 4-OHT gave an additive effect (Fig. 5D). An increased ERK signaling is reported to be part of tamoxifen resistance (5, 7). We therefore examined the total and phosphorylated forms of ERK in these cells. Higher levels of total and activated ERK were observed in LCC2 cells compared with MCF-7 cells (Supplementary Fig. S2).
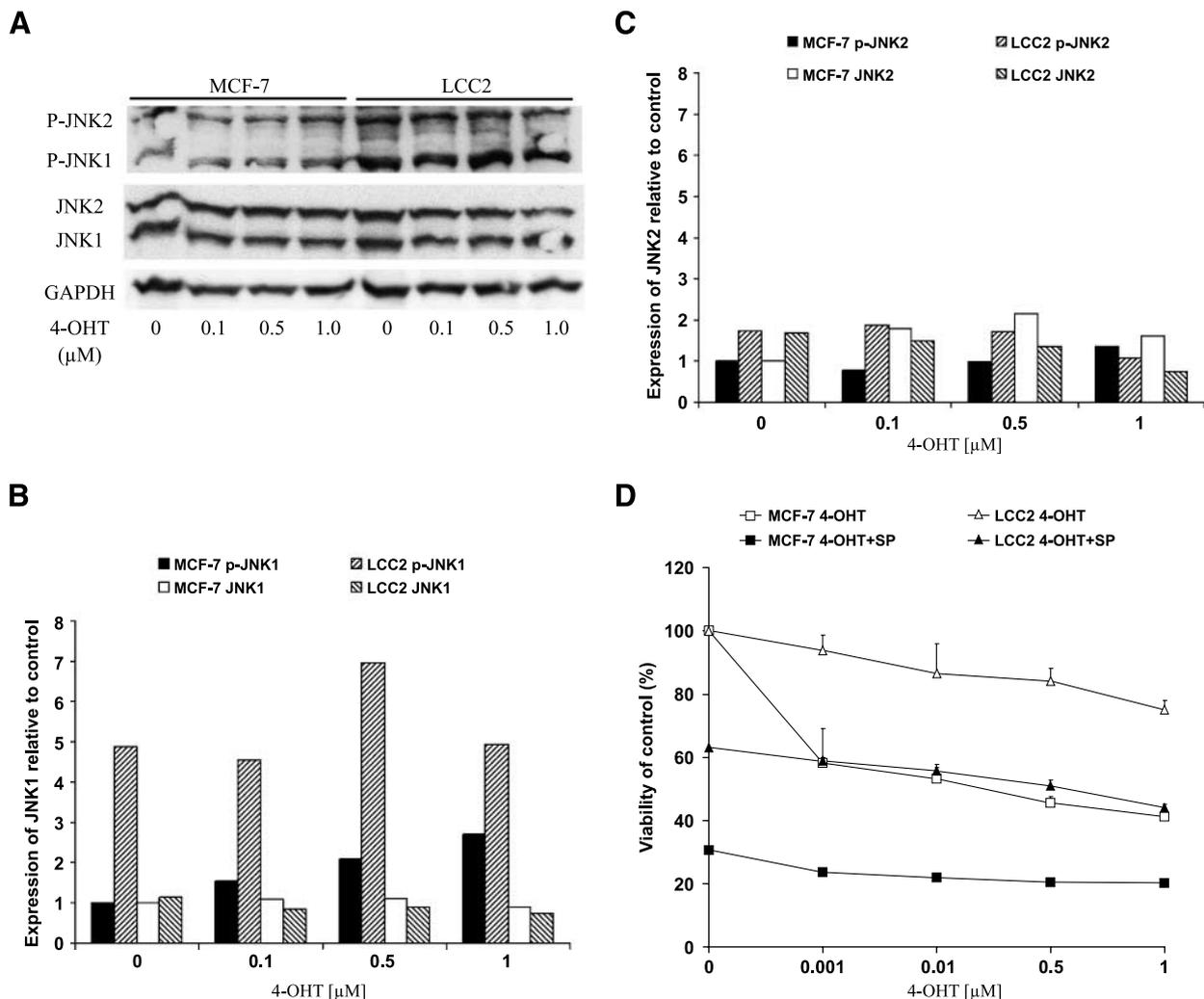
FIGURE 5. Expression of JNK in MCF-7 and LCC2 cells and the influence of JNK-inhibition on breast cancer cell survival. **A.** MCF-7 and LCC2 cells were left untreated or treated with 4-OHT at the indicated doses for 6 days and the basal and 4-OHT treatment–induced changes in total JNK expression and degree of phosphorylation of JNK were determined by Western blot analysis. **B.** Quantification of total and phosphorylated JNK1 from the Western blot in **A**. **C.** Quantification of total and phosphorylated JNK2 from the Western blot in **A**. The expressions are given as fold relative to the untreated MCF-7 cell sample after correcting for loading through glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (**B** and **C**). **D.** The effect of JNK inhibition on breast cancer cell survival and 4-OHT responses was examined by incubation with 10 μmol/L of SP600125 for 6 h before the addition of 4-OHT, as indicated. Cell viability was examined 6 days after the addition of 4-OHT using the MTT assay. Viability is given as a percentage of untreated control. Points, mean of three independent experiments with triplicate samples. The gel pictures have been cropped and full-length gels are presented in Supplementary Fig. S6.

Suppression of VEGF Signaling Down-Regulates VEGFR2 and p38 Expression

In order to address a potential cross-talk between VEGF/VEGFR2 and p38 signaling in tamoxifen-resistant breast cancer cells, LCC2 cells transfected with small interfering RNA (siRNA) against VEGF were analyzed for the expression of total as well as the activated forms of VEGFR2 and p38. Down-regulation of VEGF secretion from the cells was confirmed by ELISA assay. Secretion of VEGF was reduced by 10% to 25% of its basal level in cells transfected with negative control siRNA (Fig. 6A). In parallel, cell lysates were subject to Western blot analysis in order to examine the expression and activity of VEGFR2 and p38 upon VEGF down-regulation. In LCC2 cells, knockdown of VEGF did not have much effect on total VEGFR2 expression when examined 48 hours after transfection (results not shown); however, 72 hours posttransfection,

down-regulation of total VEGFR2 was observed (Fig. 6B and C). Using ELISA (Fig. 6B) and Western blot analysis (Fig. 6C), we found that the total VEGFR2 expression was reduced to the same magnitude (64% and 42% of control, respectively). The active form of VEGFR2 was examined by Western blot analysis using an antibody against Tyr[1214] that has been shown to be one of the major autophosphorylation sites of VEGFR2. VEGF siRNA reduced the level of phosphorylated VEGFR2 to 27% relative to control siRNA in LCC2 cells (Fig. 6B and C). Compared with the expression of p38 in cells transfected with negative control siRNA, total p38 expression was down-regulated to 63% upon knockdown of VEGF (Fig. 6D). The reduction in expression of total p38 was reflected in a similar reduction in the active, phosphorylated form of p38, even though not to the same extent as for total p38 (Fig. 6D).

Suppression of VEGFR2 Signaling Down-Regulates VEGF Secretion and p38 Expression

Down-regulation of VEGFR2 in LCC2 cells was confirmed by an ELISA (total VEGFR2) assay as well as Western blot analysis (Fig. 6B). In LCC2 cells, knockdown of VEGFR2 caused a small reduction of VEGF secretion, 86% to 90% of controls, 72 hours posttransfection (Fig. 6A). However, this reduction of VEGF secretion was not seen when MCF-7 cells were transfected with VEGFR2 siRNA (Supplementary Fig. S3). Compared with the expression of p38 in LCC2 cells transfected with negative control siRNA, total p38 expression was down-regulated to 79% upon knockdown of VEGFR2 (Fig. 6C). The reduction in expression of total p38 was reflected in a considerably reduced degree of p38 phosphorylation (53% of controls; Fig. 6D).

Discussion

In agreement with our findings on clinical materials, i.e., that tamoxifen resistance is associated with increased expression of VEGF and VEGFR2 in primary breast cancer tumors, we show here that tamoxifen-resistant LCC2 cells secrete higher amounts of $VEGF_{165}$ along with an increased phosphorylation of VEGFR2 compared with the tamoxifen-sensitive MCF-7 cells. VEGFR2 expression had earlier been suggested to be restricted to the vascular endothelium; however, more recent data has shown that VEGFR2 is also expressed in several types of nonendothelial cells including epithelial breast cancer cells (22, 23). Moreover, Weigand and colleagues have shown that VEGFR2 is activated by VEGF, and thus, is functional on the surface of breast cancer cells (24). In this study, we found that the degree of phosphorylated VEGFR2 was higher in LCC2
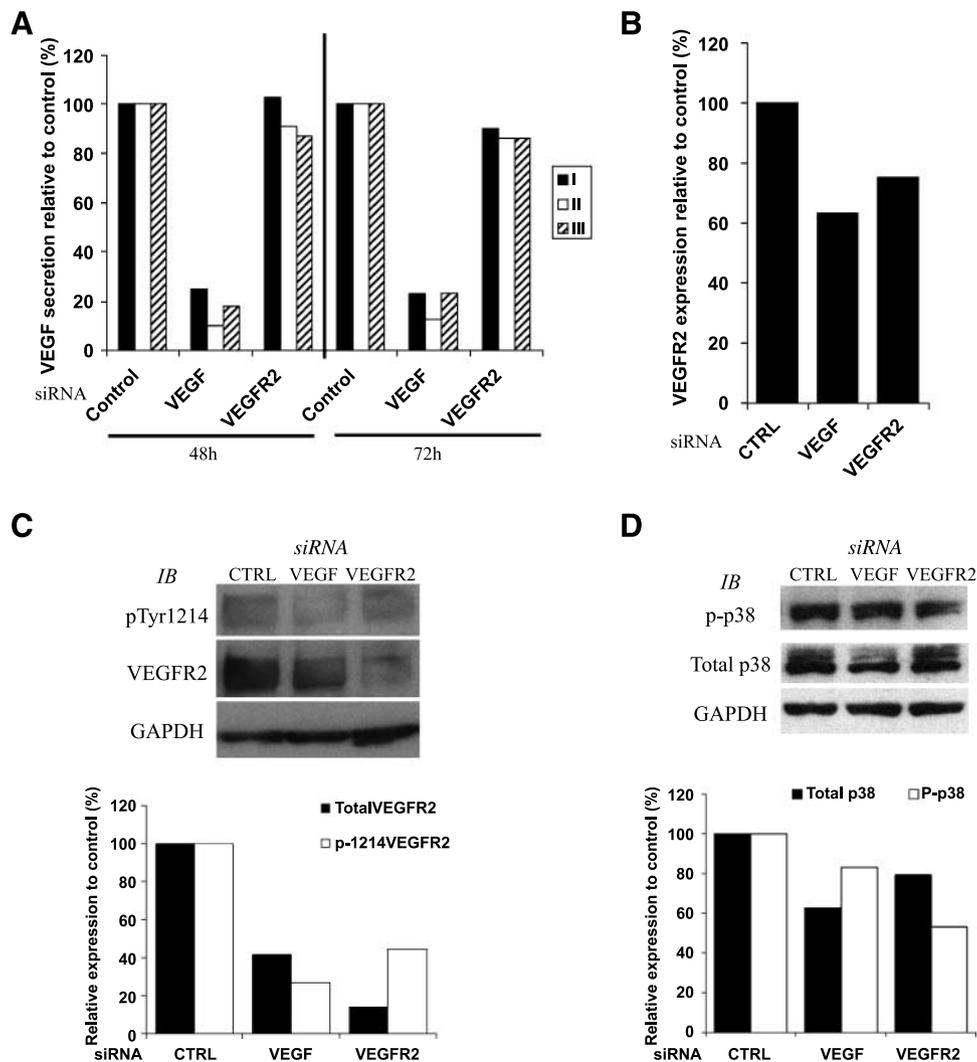
FIGURE 6. The effects of siRNA against VEGF and VEGFR2 on VEGF, VEGFR2, and p38 expression levels. LCC2 cells were transfected with siRNA against VEGF or VEGFR2. Nontargeting siRNA was used as negative control. Cells were harvested 48 and 72 h posttransfection. **A.** Knockdown of VEGF was confirmed in the three (*I-III*) experiments done using an ELISA-based assay measuring the amount of VEGF secreted from the cells. The effect of VEGFR2 knockdown on VEGF secretion was analyzed by the same assay. **B** and **C.** Knockdown of total VEGFR2 was confirmed by ELISA assay (**B**) as well as Western blot analysis (**C**). The effects of knockdown of VEGF on the expression and phosphorylation of VEGFR2 were analyzed by ELISA assay (**B**) as well as Western blot analysis (**C**). **D.** The effects of VEGF and VEGFR2 down-regulations on the expression and phosphorylation of p38 were analyzed by Western blotting. Protein levels on Western blots were corrected for loading through glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and shown as a percentage relative to control (nontargeting siRNA). **B** to **D,** the results for samples harvested 72 h posttransfection of experiment II. The gel pictures have been cropped and full-length gels are presented in Supplementary Fig. S7 (VEGFR2) and Supplementary Fig. S8 (p38).

than in MCF-7 cells. Our results suggest that MCF-7 cells have a VEGF/VEGFR2 autocrine loop for the stimulation of cell growth and that the activity of this autocrine loop is increased in tamoxifen-resistant cells compared with sensitive cells, which is in accordance with results of Scherbakov and colleagues (23). In contrast with this group, we did not find any difference in total VEGFR2 expression when comparing the two cell types, suggesting that different ways to alter VEGFR2 function should be investigated. Furthermore, we have discovered a previously unknown connection between VEGF/VEGFR2 and p38 signaling in the development of tamoxifen resistance, as shown by the reduction in the expression and activity of both VEGFR2 and p38 following siRNA-mediated down-regulation of VEGF in tamoxifen-resistant LCC2 cells. The existence of an autocrine VEGF/VEGFR2 and p38 signaling loop in tamoxifen-resistant breast cancer cells is also supported by the observation that siRNA-mediated down-regulation of VEGFR2 caused a reduction of VEGF secretion from the cells as well as a reduction in the expression and phosphorylation of p38. Increased p38 signaling has been found in a HER2-driven tamoxifen-resistant mouse model (6). Moreover, HER2 has been reported to regulate VEGF production in human breast cancer cell lines and tumors (25), and VEGF signaling has been shown to contribute to a highly proliferating, aggressive phenotype in these tumors (14, 15). However, VEGF is also expressed in a considerable number of HER2-negative tumors, suggesting that other signaling pathways may drive its expression (14, 15). One may thus speculate that increased VEGF/VEGFR2/p38 signaling may be of great importance for the growth of both HER2-positive and HER2-negative breast cancer tumors.

We show here that 4-OHT treatment of MCF-7 cells for 6 days reduced their secretion of VEGF. In addition, both the basal and active forms of VEGFR2 were significantly decreased upon 4-OHT treatment of MCF-7, partly explaining the decrease in survival of these cells after tamoxifen exposure. In LCC2 cells, the total VEGFR2 expression was not influenced by 4-OHT treatment, whereas the secretion of VEGF and the level of phosphorylated VEGFR2 was suppressed dose-dependently. However, at high 4-OHT treatment doses, both VEGF secretion and VEGFR2 phosphorylation were still evident in LCC2 cells and did not decrease below the level seen in untreated MCF-7 cells. Our results indicate that VEGF/VEGFR2 signaling is dependent on a functional ER signaling in MCF-7 cells. This may also be partly true for LCC2 cells, however, other mechanisms likely ensure that a VEGF/VEGFR2 signaling loop is kept active upon tamoxifen treatment and thus contributes to keep these cells in a proliferative state even in the absence of hormonal stimuli.

When examining the mitogen-activated protein kinases p38 and JNK, we found that these kinases were differently expressed and activated in the tamoxifen-sensitive MCF-7 and tamoxifen-resistant LCC2 cells. LCC2 cells showed increased p38 phosphorylation as well as higher total expression of p38 compared with MCF-7 cells, which is in agreement with our findings in the clinical breast cancer material.[4] Moreover, in MCF-7 cells, 4-OHT treatment activated p38, suggesting that p38 might be involved in a normal stress-activated response in MCF-7 cells because 4-OHT decreases

the viability of these cells. This is in accordance with findings by Zhang and Shapiro, who coupled the p38 activation to ER-induced apoptosis as the p38 inhibitor SB203580 largely protected MCF-7 cells from 4-OHT–induced cell death (26). Conversely, in the presence of the p38 inhibitor SB202190, we observe a decrease in MCF-7 cell proliferation. MCF-7 cells express all four p38 isoforms (α, β, γ, and δ), which have been described to have differential effects on cell proliferation (27). SB203580 has been shown to dose-dependently inhibit p38α but not p38γ proteins (28, 29). SB202190 targets p38α but not p38δ (30). Thus, the specificity of the inhibitors towards the different p38 isoforms may be part of the explanation for the inconsistency of prosurvival/prodeath responses to p38 inhibitors.

Although the total amount of p38 was slightly increased upon 4-OHT treatment of LCC2 cells, phosphorylation of p38 was dose-dependently decreased. Pharmacologic inhibition of p38 in LCC2 cells reduced cell proliferation by ∼20%, and combining SB202190 with 4-OHT treatment provided an additive effect in terms of decreased cell viability, indicating that p38 works as a survival factor in these cells. The disparity of these results might, at least in part, be explained by a divergent expression and activation of p38 isoforms in MCF-7 and LCC2 cells. Recently, Qi et al. showed that activation of the p38α isoform decreased Ras-dependent transformation through degradation of p38γ and thus stimulated stress-induced cell death (31). The p38γ isoform was found to have the opposite effect and stimulated cell proliferation independently of its own phosphorylation (31). Phosphorylation of p38α was found to be inhibited by p38γ whereas the levels of p38α protein expression remained unaltered. Because the p38γ isoform is active and stimulates cell proliferation even without being phosphorylated, the high level of total p38 in LCC2 cells might be protecting the cells against death if the p38γ is the dominant isoform in these cells. Thus, the decrease in p38 phosphorylation that we observe in LCC2 cells may hypothetically be due to the inhibitory action of p38γ on p38α phosphorylation. In MCF-7 cells, the expression of the isoforms may hypothetically be opposite to that of LCC2 cells upon 4-OHT treatment. Identifying which is the predominant active isoform of p38 in our cell model and in breast cancer patient material remains a task for future studies and may shed light on which of the p38 isoforms are essential for the development or maintenance of tamoxifen resistance.

The basal levels of total JNK were found to be similar in the two cell lines and were unaffected by 4-OHT treatment. Interestingly, we found a difference in levels of phosphorylated JNK. Although phosphorylated JNK2 was modestly increased, phosphorylation of JNK1 was highly induced in LCC2 compared with MCF-7 cells. A growth-stimulating effect of JNK has been proposed in other biological systems (32, 33). For example, increased JNK1 signaling has been shown to be part of a Ras-transformed phenotype of human small cell lung cells (32). Moreover, phosphorylation of c-*Jun*, which is required for efficient cell cycle progression, is reduced in fibroblasts depleted of JNK1, consequently resulting in a G_1-cell cycle arrest (33). Our findings are in agreement with Schiff and colleagues who showed that phosphorylation of JNK is enhanced in tamoxifen-resistant relative to tamoxifen-sensitive MCF-7 tumor

xenografts (34). The phosphorylation of JNK in this system was related to increased c-*Jun* levels and activator protein-1 activity which may induce tumor growth (34). Thus, the increased expression of phosphorylated JNK in LCC2 cells may contribute to the growth of these cells being almost unaffected by 4-OHT treatment. The striking reduction in cell viability upon SP600125 treatment (i.e., inhibition of JNK activity) further supports this idea. In summary, we find that blocking p38 or JNK activity sensitizes the tamoxifen responsiveness of MCF-7 and LCC2 cells, suggesting that a combined treatment may overcome tamoxifen resistance. Importantly, our results suggest that estrogen-independent cell growth is mediated by an autocrine release and action of VEGF causing activation of VEGFR2 and subsequently activation of p38 signaling. These findings complement targets to be inhibited for improved therapy of breast cancer patients in combination with tamoxifen treatment.

Materials and Methods

Cell Lines and Treatments

Human breast cancer MCF-7 cells and the subline MCF-7/LCC2 (referred to as LCC2) were kind gifts from Prof. Robert Clarke and Dr. Anne E. Lykkesfeldt. The LCC2 cell line has been made resistant to 4-OHT (Sigma-Aldrich) and selected as previously described (20). Both cell lines were maintained in MEM with phenol red supplemented with 10% fetal bovine serum and 1% each of L-glutamine, penicillin-streptomycin, nonessential amino acids, and sodium pyruvate (all from Sigma-Aldrich) in 5% CO_2 at 37°C. In order to reduce the influence of estrogen present in normal fetal bovine serum and the estrogen-like effects of phenol red, prior to experiments, cells were cultured for 48 h in phenol red–free MEM supplemented with 4% dextran-coated charcoal-treated fetal bovine serum (Hyclone) and the same supplements as above (medium referred to as 4% DCC-MEM).

Cell Viability Analysis

The effect of 4-OHT on MCF-7 or LCC-2 cell viability was examined using either trypan blue or 3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich). For the trypan blue assay, cells were seeded 24 h prior to treatment. Fresh medium and 4-OHT was added every second day and the cells were counted in isotonic trypan blue solution at day 0, 2, 4, 6, 8, and 10 after addition of the drug. Viability was compared with untreated controls for each time point. In MTT assay, cells were seeded at a density of 2,000 cells/well in 96-well microtiter plates and allowed to grow for 48 h. Thereafter, cells were preincubated for 6 h in the presence of 10 μmol/L p38 inhibitor SB202190 or 10 μmol/L JNK inhibitor SP600125 (both from Sigma-Aldrich) followed by the addition of 4-OHT for 144 h. The MTT assay was done according to the manufacturer's protocol. Cell viability is presented as a percentage relative to untreated cells.

Western Blot Analysis

Total protein content was extracted from cells using radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% Igepal, 5 mmol/L EDTA (pH 8.0), 0.1% SDS and freshly added 1 mmol/L phenylmethylsulfonyl fluoride, 1× PhosStop, and Mini Protease inhibitor cocktail tablets (all from Roche Diagnostics)]. Extracts were cleared from insoluble material by centrifugation. The protein content in each sample was determined using the BC Assay protein quantification kit (Uptima, Interchim). Thirty micrograms of protein from a total cell extract was loaded onto precast 10% Bis-Tris acrylamide gels in NuPage MOPS running buffer (Invitrogen Life Technology). For VEGFR2, 3% to 8% Tris-acetate acrylamide gels (Invitrogen Life Technology) were used. Proteins were transferred by electroblotting onto Hybond-C nitrocellulose or polyvinylidene difluoride membranes which were subsequently blocked in TBS-Tween (0.05%)/5% bovine serum albumin solution. Primary antibodies targeting phosphorylated p38 mitogen-activated protein kinase, p38 mitogen-activated protein kinase, phosphorylated JNK, JNK, phosphorylated ERK, ERK, and phosphorylated VEGFR2 (Tyr[1175]) were from Cell Signaling Technology, phosphorylated VEGFR2 (Tyr[1214]) was from Invitrogen, VEGFR2 was from Upstate Biotechnology, and glyceraldehyde-3-phosphate dehydrogenase was from Trevigen. The membranes were immunoblotted overnight at 4°C using primary antibodies at dilutions according to the manufacturer's directions. Membranes were probed with either mouse or rabbit horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences) for 45 min at room temperature. The resulting bands on the membranes were visualized using enhanced chemiluminescence reagents containing horseradish peroxidase substrate (Pierce). Images were scanned and quantified by Quantity One software (Bio-Rad).

siRNA Analysis

ON-TARGETplus SMARTpool Human VEGF, human VEGFR2, and siControl Nontargeting Pool (Dharmacon, Thermo Fisher Scientific, Inc.) were separately transfected into MCF-7 and LCC2 cells using the DharmaFECT4 reagent (Dharmacon) as recommended by the manufacturer. Briefly, 3.6×10^5 and 3.0×10^5 cells/well were seeded into six-well plates for 48 and 72 h of transfection, respectively. The next day, cells were transfected with 50 nmol/L of siRNA mixed with 2 μL of DharmaFECT4 and incubated for 48 or 72 h before lysis in radioimmunoprecipitation assay buffer (see Western blot procedure). Specific down-regulation of VEGF secretion by human VEGF siRNA was confirmed by ELISA assay (see below) and specific down-regulation of VEGFR2 expression by human VEGFR2 siRNA was confirmed by ELISA assay as well as Western blot analysis.

ELISA Assays

The amount of VEGF-A$_{165}$ and VEGF-A$_{121}$ secreted into the cell culture medium from MCF-7 and LCC2 cells were quantitatively measured using a Quantikine human VEGF Immunoassay (R&D Systems, Inc.). Medium from MCF-7 and LCC2 cells were collected, concentrated with Amicon Ultra-15 centrifugal filters (Millipore) and subjected to the ELISA assay according to the manufacturer's protocol. Total VEGFR2

ELISA assay (Biosource, Invitrogen) was done on cell lysates using the same amount of total protein for each sample, which was assessed using the BC Assay protein Quantification kit (Uptima, Interchim).

Disclosure of Potential Conflicts of Interest

Acknowledgments

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Reidun Aesoy, Betzabe Chavez Sanchez, Jens Henrik Norum, et al.

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